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Hilda Szabo

ABSTRACT

This study focused on high performance size exclusion liquid chromatography (HPLC-SEC) combined with two ultraviolet (UV, 254 nm and 224 nm) detection wavelengths to detect humic-like compounds and two fluorescence (FLU) excitation/emission (tyrosine-like and tryptophan-like) wavelengths to detect protein type compounds in water samples. Targeted particularly were further possibilities of this method, such as finding suitable chromatographic surrogates for organic matter and nutrient indicators for water types such as catchment surface waters, well waters, and onsite wastewater effluents, which have been studied little before. It was thus necessary to determine the optimum analytical conditions for exacting wastewater effluent analysis in term of eluent strength, eluent pH, and sample injection volume. Additionally, this study provided valuable information on the spatial and temporal behavior of dissolved organic matter along a catchment area and on the quality of onsite wastewater effluent and well water in sparsely populated areas.

A TSK-GEL G3000SW column, Na-acetate of 0.01 M at pH=7 eluent, and an injection volume of 30 μL guaranteed good separation of dissolved organic matter (DOM) in surface and well water samples up to 8 fractions and further up to 11 fractions in complex onsite black water effluents. For systematic analysis of high strength onsite wastewater effluents, we chose, based on calculations of global resolution at various eluent conditions, Na-acetate of 0.02 M at pH=7 eluent and an injection volume of 20 μL.

DOM concentration dropped along the catchment, as 35-75% of dissolved organic carbon (DOC) was eliminated. DOM in drains had up to 80% high molecular weight (HMW) fraction and lakes only 50-60% HMW. Drains had high DOC in summer and lakes in winter and spring with seasonal increase in DOC resulting from increased HMW fractions in these waters. The water treatment plant eliminated HMW fractions from raw water up to 100%, intermediate MW (IMW) fractions up to 87%, and low LMW fractions up to 66%. A seasonal increase in raw water DOM was detected in drinking water samples as increased IMW and appearance of HMW fractions. Of the two protein-type detections, tryptophan-type signals were clearly measured in surface water. Tryptophan-like FLU, as sum of peak

height (SPH), was consistently higher in the drain affected by agriculture than in the drain in the mire area.

The study on well waters showed that, on average, shallow and deep well water differ little in quality in the sparsely populated agricultural areas studied. According to HPLC-SEC-UV254, high-DOC well water samples had clear and often dominant HMW fractions and low-DOC samples hardly any HMW fractions but dominant IMW fractions. The LMW fraction, correlating with nitrate, indicates anthropogenic influence. Nitrate was precisely calculated from the peak height (PH) of the LMW fraction detected by UV-224.

Our study on onsite blackwater effluent (BWE) and greywater effluent (GWE) disclosed the overall quality of onsite wastewater effluents with BWEs having higher mean values than GWEs for all the conventional indicators measured. The chromatograms (UV-254, tyrosine, and tryptophan) of onsite wastewater effluents showed the regular peaks for surface and well waters and extra peaks eluted over the permeation volume. Dividing the chromatograms into 3 regions helped identify the best possible surrogates for conventional indicators. Region 3 comprising the late peaks eluted over the permeation volume in the tyrosine- and tryptophan-chromatograms correlated best with biochemical oxygen demand (BOD-7), showing that these fractions are biodegradable. Tyrosine-like chromatograms assess best DOC and BOD-7, trytophan-like chromatograms best total nitrogen (TN), and UV254 and tyrosine-like chromatograms best the chemical oxygen demand (COD) of wastewater effluents. Regression equations corresponding to the best correlations between the chromatographic and conventional indicators are given in the study for reliable calculation of DOC, COD, and BOD-7 and rough assessment of the TN.

This study highlights the fact that secondary interactions, unwanted in SEC can be exploited in nitrate measurement of well waters and BOD assessment of high strength wastewater effluents.

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ABBREVIATIONS

A224, 254, 280, 416	Absorbance at 224nm, 254nm, 280 nm, 416 nm
BOD-5; BOD-7	5-and 7 days biochemical oxygen demand
BWE	blackwater effluent
COD	chemical oxygen demand
dEfOM	dissolved effluent organic matter
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
DOM	dissolved organic matter
ex/em	excitation-emission
EEMS	excitation emission matrix fluorescence spectroscopy
FLU	fluorescence
GWE	greywater effluent
HMW	high molecular weight
HPLC-SEC	high performance liquid size exclusion chromatography
HPI	hydrophilic
HPI-A	hydrophilic acid
HPI-B	hydrophilic base
HPI-N	hydrophilic neutral
HPO-A	hydrophobic acid
HPO-N	hydrophobic neutral
IMW	intermediate molecular weight
IS	ionic strength
LMW	low molecular weight

M_n	number-average molecular weight distribution
MS	mass spectrometry
M_w	weight-average molecular weight distribution
MTPE	municipal treatment plant effluent
MWD	molecular weight distribution
MWp	molecular weight corresponding to peak maxima
NOM	natural organic matter
PA	peak area
PH	peak height
Rt	retention time (of peak maxima)
SFS	synchronous fluorescence scanning
SPA	sum of peak area
SPH	sum of peak heights
SUVA	specific ultraviolet absorbance
TOC	total organic carbon
TN	total nitrogen
TON	total organic nitrogen
TP	total phosphorous
Tryp	tryptophan
Tyr	tyrosine
UF	ultrafiltration
UV/VIS	ultraviolet/visible
XAD	highly adsorbent resins used in organic matter fractionation
WFD	Water Framework Directive
WHO	World Health Organization
WWE	wastewater effluent
WWTP	wastewater treatment plant

ORIGINAL PUBLICATIONS

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Author's contribution

Publication I I planned the experiments together with prof. Tuula Tuhkanen, performed most of the experiments, and drafted the article manuscript, which was finalized by all the authors.

Publication II I planned the experiments together with the co-authors, performed all the experimental work, and drafted the manuscript, which was finalized by the co-authors.

Publication III I planned the experiments together with prof. Tuula Tuhkanen, performed all the experiments, drafted the manuscript, and finalized it together with Dr. Lepistö.

Publication IV I planned the experiments, performed most of the experiments, drafted the manuscript, and finalized it with Dr. Lepistö.

1 INTRODUCTION

Water is a continuously cycling substance that follows the pattern of the hydrological cycle powered by the sun. A small and polar compound, water interacts with every compound that it comes in contact with. Therefore, pure water as such does not exist in the environment. Naturally, waters found in different environmental settings vary in their composition, reflecting the media characteristics of their surroundings (Backman et al., 1998; Korkka-Niemi, 2001).

Of all the water on the earth, 97.5 % is salty water found in oceans and seas, and 2.5 % is "freshwater," which contains significantly less dissolved salts. However, because most freshwater is in the form of ice (1.7 %), which is unavailable for human activities, the term "freshwater" signifies the remaining 0.8 % of water found on our planet that is in the form of groundwater or surface freshwater (rivers, lakes) (Manahan, 2007).

The surrounding environment greatly affects the quality of freshwater. Groundwater usually contains less dissolved organic matter (DOM), fewer microorganisms, and more dissolved salts than surface waters. Additionally, freshwater quality is affected by a series of natural processes, classified as hydrological (e.g., dilution, evaporation, percolation, settling), physical (e.g., gas exchange, adsorption/desorption, volatilization, diffusion), chemical (acid-base, redox, ion exchange processes, precipitation, photodegradation), and biological (primary production, growth, die-off, biodegradation, bioaccumulation, biomagnification) (Strobl & Robillard, 2008).

Since the industrial revolution, human activities have been markedly bearing on freshwater quality. For example, improper water management practices and land use and release of water, air, and soil contaminants by industrial and other human activities are all threatening the aquatic systems (Strobl & Robillard, 2008). Furthermore, markedly increased DOM concentrations have been detected in the northern hemisphere since the early 1990s, a phenomenon that is not yet fully understood (Filella & Rodriguez-Murillo, 2014, Creed et al., 2018). Several factors,

such as changes in land use, air temperature, increased precipitation, increased atmospheric carbon dioxide, atmospherically deposited nitrogen accumulation, atmospheric sulfur, and sea salt deposition have been studied as contributors to this phenomenon (Monteith et al., 2007; Sucker & Krause, 2010, Butturini et al., 2016). The only influencing factor was found to be anthropogenic sulfur and sea salt deposition, whose rate of decrease since the 1990s has been proportional to the increase in DOM in surface waters (Monteith et al, 2007).

One possible source of contamination is wastewater or purified wastewater effluents released into surface water bodies or percolated through subsoil into groundwater. According to Eurostat (2018a and 2018b), in 2015 in 21 European countries, an average of 78.2 % of population were connected to urban centralized wastewater treatment and an average of 9.2% to another type, mostly decentralized, onsite wastewater treatment. These figures suggest that the raw wastewater/excrements of about 10-15% of population continue to be released untreated into the environment. These waters contain pathogens, organic matter, and nutrients, such as phosphorous and nitrogen, which are responsible for surface water eutrophication.

Wastewater from dwellings not connected to a sewage network is usually treated onsite with septic systems as primary wastewater treatment (settling of solids). Usually, such a system consists of a septic tank and a drain field. The septic tank ensures a certain residence time for the incoming wastewater (usually > 36h), during which the water is purified anaerobically. Most heavy and light solids separate and settle on the bottom of the tank, and organic matter is transformed (mineralized) mostly by anaerobic microorganisms. Of low quality, the septic tank effluent is further purified via mechanisms, such as biotransformation, straining, sorption, plant uptake by percolation into the soil through a drain field, and soil trapping (Van Cuyk et al., 2001). The grade of purification depends on the activity of the biomat formed slowly in the upper part of the infiltration area (Van Cuyk et al., 2001; Gill et al., 2009; O'Luanaigh et al., 2012). A balance between the rates of biomat formation and percolation ensures a good removal of organic matter and bacteria (over 90%) but only a limited removal of nitrogen (Van Cuyk et al., 2001). The percolating effluent of unknown final quality most often ends up in groundwater or is collected and released directly into a surface water stream.

Problems with operating septic systems often include faulty drainage field design, poor maintenance, and improper location of the septic system (Butler & Payne, 1995;

Middle, 1996). Consequently, impaired septic effluents can negatively impact on the quality of the ground or stream water receiving them. In fact, studies show a negative effect of onsite septic systems on the quality of water in wells located near or downstream from (Reide Corbett et al., 2002; Szabo et al., 2009), or at sites with highly permeable subsoils (Gill et al. 2009; O'Luanaigh et al., 2012; Morrissey et al., 2015; Phillips et al., 2015). According to Withers et al. (2012), during low-flow summer periods, septic systems can significantly increase nutrient concentrations in streams and thus contribute to riverine eutrophication to an extent greater than previously assumed. Better design, control, and maintenance, and application of some alternative treatment systems have been offered as options to improving onsite wastewater treatment (Butler & Payne, 1995; Middle, 1996; Withers, 2012).

In addition to the above negative effects, both untreated and purified wastewater effluents contain emerging microcontaminants, such as antibiotics, hormones, endocrine disrupting chemicals, X-ray contrast media, and pharmaceuticals (Jenssen et al. 2010; Eveborn et al., 2012; O'Launaigh et al., 2012, Garcia et al., 2013; Pal et al., 2014; Sclar et al., 2016; Bieber et al., 2018; Sousa et al., 2018). As a consequence of water cycling, these compounds regularly occur in surface water and even well water and municipal drinking water (Kolpin et al., 2002; Barnes et al., 2008; Carrara et al., 2008; Phillips et al., 2015; Subedi et al., 2015; Schaider et al., 2016). To eliminate these compounds, they should be replaced with ecologically friendly alternatives, and/or new wastewater and drinking water treatment technologies should be developed to remove them altogether (Pal et al., 2014; Bieber et al., 2018).

Water quality is determined by water quality indicators, divided into physical, chemical, and biological to describe some physical characteristic of water, the amount of some chemical compound, or the presence and amount of some microorganism in water. Water quality indicators are divided into two main categories: health-based indicators set mainly for drinking water to prevent the negative effects of contaminated water on human health and ecological indicators set for surface waters to assess the ecological state of a water body. These indicators are presented as required values, guidelines, or targets by official bodies of the European Union (WFD, 200/60/EC; WHO, 2011; Council Directive 98/83/EC). Health-based guideline values for drinking water are given for a great number of water quality indicators: microorganisms, heavy metals and some nonmetals, some synthetic organic chemicals used in agriculture or industry (e.g., pesticides, organic solvents, disinfectants), and radioactive isotopes (radionuclides) (WHO, 2011). The

quality requirements for urban wastewater effluents in the EU are given by Council Directive 91/271/EEC, whereas those for onsite wastewater effluents are given on the national level. These requirements are set for five effluent quality indicators describing organic matter content, suspended solids, and nutrient content such as nitrogen (N) and phosphorous (P): 5-day biochemical oxygen demand (BOD-5), chemical oxygen demand (COD), total suspended solids (TSS), total phosphorous (TP), and total nitrogen (TN) (91/271/EEC).

The above indicators are, however, sum parameters and provide no information on the components of organic matter. Moreover, BOD and TP measurements are time consuming whereas TN and COD measurements use harmful chemicals; therefore, alternative water quality indicators and analytical methods are needed. High performance size exclusion liquid chromatography (HPLC-SEC) with multiple detections could be one suitable alternative method: it is fast, uses no toxic chemicals, and separates the mixtures of organic matter into components that are detectable by ultraviolet/visible (UV/VIS) or fluorescence (FLU) detection. This study assesses the possibilities of HPLC-SEC to analyze various water samples for quick and reliable information on water quality and contaminant amounts.

2 BACKGROUND

2.1 Organic matter, nitrogen, and phosphorous in water and their conventional analysis

The term *dissolved organic matter* (DOM) represents the total amount of organic compounds dissolved in water with *dissolved* understood conventionally as all those compounds that pass through a 0.45-μm pore size filter. Organic compounds are ubiquitous in all types of water samples: drinking water, groundwater, surface water, wastewater, and wastewater effluents (Matilainen et al., 2002; Mitikka et al., 2005; Leenheer and Croué, 2003; Michael-Kordatou et al., 2015). In drinking and freshwater, DOM can cause odor and color problems, in freshwater it contributes to the transport of hydrophobic synthetic organic pollutants and heavy metals, and in water treatment plants it is a source of unwanted disinfection by-products (Leenheer and Croué, 2003; Michael-Kordatou et al., 2015). Consequently, aquatic DOM is of main research and regulatory interest. Because nitrogen and phosphorous are the nutrients necessary to ensure the growth of autotrophic organisms, they are thus responsible for the eutrophication of surface waters. The main cause of the eutrophication of inland waters is their increased phosphorous concentration, caused by excessive inputs from agricultural runoffs and sewage discharges (Correll, 1998; Carpenter, 2005). These inputs also add to the ammoniacal nitrogen content of surface waters, this form of nitrogen being toxic to all vertebrates (Randall & Tsui, 2002). Moreover, in the form of soluble nitrate ion (NO_3^-) nitrogen infiltrates into ground water from agricultural fields, posing a serious health threat, the blue-baby syndrome, in small children. In the presence of electron donors, such as organic matter, ferrous ion, and low dissolved oxygen content, nitrate can in groundwater attenuate due to biological denitrification; however, the required conditions thereto are site-specific and seasonal (Clay et al., 1995; Thayalakumaran et al., 2008). Therefore, DOM, N, and P are the routinely measured indicators to monitor surface water, groundwater, and wastewater effluent. (Strobl & Robillard, 2008; 98/83/EC; 91/271/EEC)

The conventional water quality indicators that describe DOM in drinking and surface water are color, COD, total organic carbon (TOC), and dissolved organic carbon (DOC); they describe the total amount of organic matter in the sample. Additionally, for wastewaters and wastewater effluents, BOD indicates the biodegradable part of organic matter (WHO, 2011; 98/83/EC; 91/271/EEC). These indicators are sum parameters that reliably describe the total organic content but provide no information on particular components of DOM. Additionally, COD measurements require harmful chemicals, whereas BOD determination is time consuming, from a minimum of 5 days on. Routine measurement of N comprises Kjeldahl analysis of organic-N and ammonia-N, separate spectrophotometric measurement of nitrate-N (NO_3-N) and nitrite-N (NO_2-N), and assessment of total organic nitrogen (TON) as a difference between Kjeldahl and nitrate/nitrite-N content. Phosphorous is measured spectrophotometrically (91/271/EEC). Because Kjeldahl analysis is laborious and requires harmful chemicals, and because phosphorous measurement is lengthy, alternative methods are being searched to replace or complement these conventional indicators (Prasse et al., 2015; Zulkifli et al., 2018).

2.2 DOM composition of different waters

In aquatic environments, the greatest fraction of DOM is natural organic matter (NOM). A mixture of complex macromolecules, NOM is the end product of the rapid biodegradation of terrestrial and aquatic plants and has a refractory character in that it continues to biodegrade further very slowly (Leenheer & Croue, 2003). It has various functions in the environment in that it can serve as a ligand in the complexation of metals and can adsorb xenobiotic compounds and facilitate their transport in aqueous environments. It can itself adsorb to mineral surfaces and settle in soil or sediments and can be partially oxidized and assimilated by microbes (Frimmel, 1998). In water treatment, NOM is not desired, because it can release color and odor to water, and because it is oxidized by chlorine used in water disinfection, it produces undesirable disinfection byproducts (Zsolnay, 2003; Frimmel, 1998).

The scientific community has long aimed, unsuccessfully, to reveal the composition and structure of NOM macromolecules. NOM/DOM is frequently characterized by

fractionating and isolating the organic matter from water, a labor-intensive and long procedure exploiting mostly XAD-8 hydrophobic resin, followed by cation- and anion-exchange resins, and subsequently extracting the retained fractions. The separation is done at extreme pH values, which alter the structure of organic molecules and bias their further characterization. Depending on the procedure, DOM can be separated into three fractions: humic acids, fulvic acids, and hydrophilic acids (HPI-A) (Gron et al., 1996; Artinger et al, 2000; Kumke et al., 2001) or into 5 or more fractions: hydrophobic acids HPO-A, hydrophobic neutrals HPO-N, hydrophilic bases HPI-B, hydrophilic acids HPI-A, and hydrophilic neutrals HPI-N or their sub-fractions (Mattson & Kortelainen, 1998; Leenheer and Croue, 2003). The fractions are characterized by various methods, such as high performance liquid size exclusion chromatography (HPLC-SEC), UV/VIS or FLU spectroscopy, or they are further degraded to detect the building blocks of component macromolecules (Gron et al., 1996; Mattson & Kortelainen, 1998; Brinkmann et al., 2003). Frimmel (1998) identified 17 amino acids (dominant: aspartic acid, cysteine, and leucine) and several carbohydrates (dominant: glucose, galactose, mannose, and xylose) in acid hydrolyzed environmental NOM samples. Gron et al. (1996) found aliphatic-C, carboxyl-C, carbohydrate-C, carbonyl-C, aromatic-C, amino acids, chlorine, bromine, iodine, and sulfur in groundwater DOM fractions. Brinkmann et al. (2003) identified formic, acetic, pyruvic, oxalic, malonic, and succinic acids as a result of the photodegradation of hydrophilic DOM fractions of surface waters. The hydrophobic DOM fraction resisted degradation better than the other fractions (Frimmel, 1998; Brinkmann et al., 2003)

Because groundwater DOM originates from subsurface organic deposits or leaches from upper soils, its composition depends mostly on its soil, peat, or marine origin. Compared to terrestrial DOM, marine DOM has a higher amino acids/carbohydrates ratio and more iodine and bromine, whereas terrestrial DOM is more aromatic (Gron et al., 1996; Artinger et al, 2000). Groundwater conditions affect DOM as well so that reducing conditions lead to a high S content, and old source rock leads to a low carbohydrate DOM content, whereas a high calcium concentration removes humic acids from the aqueous phase (Gron et al., 1996).

In surface waters, the two main NOM groups are allochthonous NOM, originating from terrestrial plants, and autochthonous NOM, produced from algae, bacteria, and macrophytes in aquatic environments (Leenheer & Croue, 2003). These groups have distinct characteristics: allochthonous NOM has more glucose and xylose, whereas

autochthonous NOM produces more deoxysugars and amino carbohydrates during acid hydrolysis, allochthonous NOM has a higher molecular weight distribution (MWD) and higher aromaticity (expressed as specific ultraviolet absorbance SUVA) and is less degradable/biodegradable than autochthonous NOM (Frimmel, 1998; Rosario-Ortiz et al, 2007). Lake waters high in DOC have a high proportion of hydrophobic content, whereas those with low DOC have a high proportion of hydrophobic fractions (Mattson & Kortelainen, 1998). According to Ma et al. (2000), fulvic acids predominate in surface waters.

Dissolved effluent organic matter (dEfOM) found in wastewater effluents contains more hydrolysable fractions than groundwater and surface water DOM (Frimmel, 1998). dEfOM is a mixture with an extremely high number of molecules grouped as (1) soluble microbial products (SMP), which constitute the greatest proportion of dEfOM and originate from bacterially decomposed organic substrates and cell lysis of decayed biomass; (2) NOM from drinking water, and (3) trace organic compounds found in ng/L or μg/L amounts, such as endocrine disrupting chemicals, pharmaceuticals and personal care products, and disinfection byproducts (Her et al., 2003; Michael-Kordatou et al., 2015). XAD-fractionated dEfOM contained a low amount of humic acids with a dominant HPI fraction (Ma et al., 2000). Also detected in dEfOM were surfactants found in detergents (linear alkyl benzene sulfonates and sulfophenyl carboxylic acid) (Wang et al., 2018). dEfOM is characterized as emitting intense protein-like FLU, which is seen in receiving surface waters as well (Rosario-Ortiz et al., 2007; Baker et al., 2004).

2.3 Trends in water analysis

The techniques used to monitor water can be classified according to different criteria: in-line sensor-based methods versus discontinuous sample-based methods and physical, chemical, or biological methods (Zulkifli et al., 2018). An important study domain is fecal source tracking, which aims at detecting and identifying animal versus human sources of fecal influence on a freshwater sample. Deoxyribonucleic acid-based (DNA) identification using polymerase-chain-reaction(PCR)-based molecular methods (Field & Samadpour, 2007; McLellan & Eren, 2014; Silva & Dominguez, 2015; Hering et al, 2018; Zulkifli et al. 2018) and analysis of some chemicals, such as caffeine, fecal sterols, bile acids, bleaches, fragrances, pesticides,

and polycyclic aromatic hydrocarbons, (PAH) can be used to detect sources of fecal contamination. However, the chemicals' behavior in the environment may lower their correlation with pathogens (Field & Samadpour, 2007). Another important domain is the development of on-line detection of contaminants, both microbiological and chemical. The most promising methods are microfluidic sensors (Zulkifli et al. 2018), spectroscopic techniques (Lopez-Roldan et al., 2013; Zulkifli et al, 2018), and biosensors (Zulkifli et al, 2018; Jiang et al, 2018). The studies have, however, mentioned several disadvantages in the above methods. For example, inadequate detection sensitivity and weak sensor response as well as sensitivity to a bulk environment compromise low-concentration measurements. In addition, samples must be pre-treated for microfluid measurements and some spectroscopic methods, which hikes up analysis costs (Lopez-Roldan et al., 2013; Zulkifli et al, 2018; Jiang et al, 2018). Discontinuous sample-based methods are the usual laboratory methods focused on bacterial or chemical analysis. Advances have been made with DNA amplification and fluorescence in-situ hybridization methods, which allow identification and quantification of a series of bacteria and viruses in water samples; however, these methods remain time consuming and poorly sensitive to low concentrations of microorganisms (Zulkifli at al., 2018).

Analysis of emerging contaminants (micropollutants) in environmental water samples has been an important domain in water analysis. Appearing in water samples in μg/L or ng/L amounts, these contaminants are surrounded by a complex matrix, which makes their analysis challenging. Depending on the properties of the analyte, the preferred methods for their analysis are liquid or gas chromatography coupled with a mass spectrometer (MS) as detector (Farré et al., 2012; Dujakovic et al., 2010). Before analysis, samples must be pre-concentrated via solid phase extraction (SPE), which consumes high volumes of organic solvents and is time consuming (Plotka-Wasylka et al, 2015).

As for gross organic water quality indicators, the focus has been (1) to search for BOD surrogates that would lead to faster BOD assessment and (2) to develop methods that would allow characterization of particular components of DOM. For BOD measurement, biosensors and microbial fuel cells are the most promising techniques, for they can assess BOD in about 30 min; however, their sensitivity and reproducibility are compromised by the sensitivity of microbes to toxic shocks (Jouanneau et al., 2014; Jiang et al., 2018).

2.4 Characterization of particular components of DOM in water by UV/VIS and FLU spectroscopy

To characterize DOM components, most studies focus on three analytical methods: UV/VIS spectroscopy, fluoresce spectroscopy (FLU), and HPLC-SEC combined with ultraviolet (UV), fluorescence (FLU), or DOC detection (Leenheer & Croue, 2003; Wu et al., 20017a; Bhatia et al., 2013; Her et al., 2002; Lankes et al., 2009)

2.4.1 UV/VIS spectroscopy

UV/VIS spectroscopy is based on the property of organic molecules to absorb electromagnetic radiation in the UV/VIS range. This technique is useful in characterizing DOM both quantitatively and qualitatively (Li & Hur, 2017; Korshin et al., 2018). Samples are scanned for a range of UV/VIS spectra, usually for a wavelength ranging above 230 nm to avoid the interference of nitrate (Korshin et al, 1997; Uyguner & Beckbolet, 2005). The extracted spectral data, such as the area below 250-350 nm or absorbance at 254 nm (A254) or 280 nm (A280) approximate COD and DOC for aquatic organic matter isolates (Weishaar et al., 2003), surface waters and drinking waters (Vuorio et al., 1998; Kalbitz et al., 2000; Hur et al., 2006; Liu et al., 2010, Boghoth et al. 2011), wastewater effluents (Wu et al., 2006; Wu et al, 2006; Korshin et al., 2018) and well waters (Szabo & Tuhkanen, 2016), wastewater influents, and urine (Wu et al., 2006; Louvet et al, 2013; Yang et al., 2015). From spectral data, parameters such as SUVA-254 and Molar Absorbance-280 or absorbance ratios (A254/A365 or A254/A436) can be calculated. These ratios have been used to assess the aromaticity of DOM and to determine its autochthonous versus allochthonous origin (Chin et al. 1994, Peuravuori and Pihlaja, 1997, Frimmel and Abbt-Braun, 1999, Matilainen et al., 2011). In addition, A224 was useful in estimating simultaneously high NO_3^- concentrations in well waters (Szabo & Tuhkanen, 2016). The drawback of UV/VIS spectroscopy, especially in in-situ monitoring, is that changes in water temperature and increases in turbidity affect UV/VIS absorbance, leading to miss-estimation of samples' organic matter content (Lee et al., 2015). Moreover, changes in pH affect UV/VIS absorbance as well, causing it to decrease with decreasing pH (Spencer et al., 2007b).

2.4.2 FLU spectroscopy

FLU spectroscopy is a non-destructive technique that allows scanning of whole water samples for rapid DOM detection. It is based on the characteristics of DOM to become fluorescent when high energy UV light hits moieties of DOM called fluorophores. Excited fluorophores then emit low-energy UV/VIS light (Hudson et al, 2007), and recorded spectra allow estimation of the amount and nature of DOM (Carstea et al., 2016 and literature within).

Literature reviews cite the following FLU techniques for water and wastewater analysis:

1. use of a single excitation-emission (ex/em) wavelength pair to detect a specific molecule,
2. FLU emission spectrometry, where a single excitation wavelength from the UV region is used, and the emission spectra are recorded,
3. synchronous FLU scanning (SFS), where FLU intensity is measured at emission wavelengths maintaining a constant value of $\Delta\lambda = \lambda_{em} - \lambda_{ex}$ between 12-60 nm,
4. ex/em matrix FLU spectroscopy (EEMS), where ´contour maps´ are constructed from simultaneous excitation and emission FLU intensity scans over a wavelength range. An advanced form of EEMS is to further deconvolute the EEMS scans with mathematical models, such as parallel factor analysis (PARAFAC), into components that, e.g., could surrogate conventional water quality indicators. (Hudson et al, 2007; Carstea et al., 2016)

FLU spectroscopy has been used in numerous studies to characterize humic matter and fractionated aquatic NOM (Hautala et al., 1999; Peuravuori et al., 2002; Chen et al., 2002; Chen et al., 2003; Kim et al., 2006), DOM in rivers and lakes (Chen et al., 2003; Belzile & Guo, 2006; Baker et al., 2007; Spencer et al., 2007a; Spencer et al, 2007b; Wu et al, 2007b; Lee et al., 2015), and DOM in ground waters (Kalbitz et al., 2000). In addition, it has been used to monitor organic matter removal in drinking water treatment plants (Boghoth et al., 2011). However, the most promising applications have been in wastewater analysis to characterize wastewater influents (Wu et al., 2006; Yu et al. 2013), urine (Wu et al., 2006), wastewater effluents (Chen et al., 2003; Henderson et al., 2009; Baker et al., 2004; Wu et al., 2006; Yang et al., 2015), farm wastes (Baker, 2002), and wastewater sludge extracellular polymers (Sheng & Yu, 2006). Moreover, organic matter removal during membrane treatment of wastewater (Galinha et al., 20012) and slaughterhouse wastewater biodegradation

(Louvet et al., 2013) has successfully been studied by FLU spectroscopy. WWEs have characteristic FLU spectra that allow their fingerprinting in surface waters. SFS was used by Galapete et al. (1997) to detect sewage effluents in a river downstream to a WWTP and by Wu et al. (2006) to identify urine in raw sewage. FLU emission spectra were used to show an alteration in DOM along a stream, where FLU originating from WWE persisted for a short time along the river (Wu et al., 2007a). In several studies, EEMS as such or combined with UV/VIS spectroscopy was used to trace WWE in surface waters (Baker, 2002; Chen et al., 2003; Baker et al., 2004; Henderson et al., 2009). These studies have confirmed that SFS and EEMS can be used to determine the origin of DOM (Spencer et al., 2007a; Yang et al., 2015). In water analysis, two main classes of fluorophores have emerged: "humic-like" fluorophores and "protein-like" fluorophores (Table 1, Table 2). It is generally assumed that long wavelength fulvic-like/humic-like FLU is due to fused-ring aromatic moieties of recalcitrant DOM (Leenheer & Crue, 2003; Hudson et al, 2007), whereas short wavelength FLU is generated by structurally simple groups with electron donating substituents, such as –OH, $-NH_2$, and $-O-CH_3$ (Kalbitz et al., 2000; Peuravuori et al., 2002). Natural waters abound with predominantly fulvic-like and humic-like FLU compositionally characteristic of the source (Kalbitz et al., 2000; Peuravuori et al., 2002; Chen et al., 2002; Belzile & Guo, 2006; Kim et al., 2006; Spencer et al., 2007a).

Protein-like FLU is attributed to three fluorescent amino acids, tryptophan, tyrosine, and phenylalanine (Table 1), and is related to the activity of bacteria and their bioavailable substrate (Hudson et al., 2007). In the above studies (Table 2), wastewaters showed both humic-like and intensive protein-like FLU. Furthermore, protein-like FLU, which is biodegradable, has been shown to be more efficiently removed in wastewater treatment than fulvic-like FLU, yet WWEs contain clearly measurable amounts protein-like FLU (Saadi et al, 2006; Wu et al, 2006; Boghoth et al., 2011; Louvet et al. 2013; Yu et al, 2013). Once released into the environment, WWEs cause a brief increase in protein-like FLU in surface waters, which dissipates faster than fulvic-like FLU (Wu et al., 2007b). Nevertheless, this protein-like FLU is still useful in detecting sewage-origin anthropogenic impacts in surface waters (Galapate et al., 1997; Baker, 2002; Chen et al., 2003; Baker et al., 2004; Wu et al., 2007b; Spencer et al., 2007a; Henderson et al., 2009; Lee et al., 2015; Yang et al., 2015). However, no studies are available on the applicability of FLU spectroscopy to detecting anthropogenic influence on groundwater.

Table 1. Structure of the fluorophores that cause DOM fluorescence

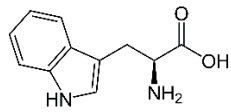
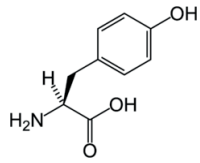
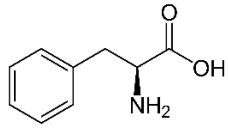
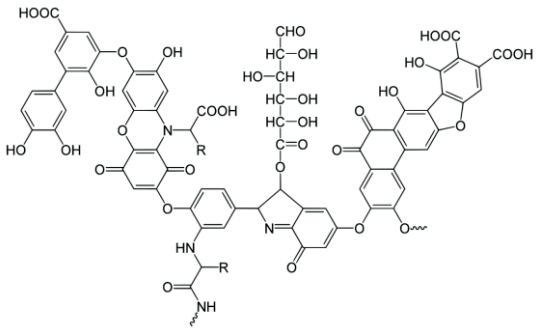
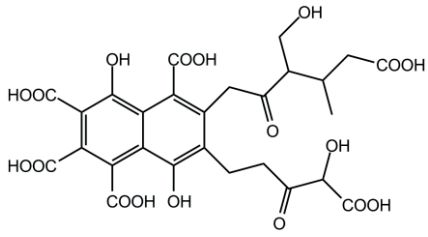
Name	Structure
Tryptophan	
Tyrosine	
Phenylalanine	
Humic acid (model structure)	
Fulvic acid (model structure)	

Table 2. DOM fluorophores and their detection wavelengths

Humic-like fluorophores	**λ_{ex}/ λ_{em}**	**Reference**
humic-like	330-350/420-480 250-260/380-420	Leenheer & Crue, 2003
marine humic-like	310-320/380-420	Leenheer & Crue, 2003
fulvic-like	320-340/410-430	Baker, 2001
humic-like	370-390/460-480	Baker, 2001
fulvic-like	337/423	Her et al., 2003
humic/fulvic-like	249-281/434-443 330-339/430-437	Saadi et al., 2006
fulvic-like	380/430	Wu et al., 2006
fulvic-like	355/390	Kalbitz et al., 2000
Protein-like fluorophores	**λ_{ex}/ λ_{em}**	**Reference**
tyrosine-like	270-289/300-320	Leenheer & Crue, 2003
tryptophan-like	270-289/320-350	Leenheer & Crue, 2003
tryptophan-like	275/350	Baker, 2001
tryptophan-like	220/350 280/350	Baker et al., 2004
protein-like	281/348-359 281/346-359	Saadi et al., 2006
protein-like	278/353	Her et al., 2003

One problem associated with FLU spectroscopy is the so-called "inner filtering" effect in concentrated samples such as high DOM-containing surface water samples, wastewaters, and wastewater effluents. Various fluorophores can absorb emitted light, leading to a long emission wavelength bias in analysis (Hudson et al, 2007). A further effect on FLU measurements is light scattering by colloids present in unfiltered samples (Hudson et al., 2007; Lee et al, 2015). In EEMS, the Raman line is apparent at 260-350/280-400 nm as a consequence of the vibration of O-H bonds in the water molecule when irradiated with UV light, which can obscure tyrosine-like FLU in spite of correction by spectral subtraction (Hudson et al, 2007)

The composition of fluorophores changes in time and must be taken into account in periodic sampling or online monitoring (Lee et al, 2015). The factors with a significant effect on ex/em wavelength and FLU intensity are pH, quenching by metal ions, and change in temperature. Freshwater sample FLU, and especially tryptophan-like FLU, decreased with decreasing pH in surface water samples (Baker et al., 2007; Spencer et al., 2007). Metals normally found in freshwaters (Fe, Al) and others possibly found in wastewaters (Cu, Pb, Cr) quenched fluorophores by forming complexes (Hudson et al., 2007). Increased temperature caused collisional quenching, which lowered FLU intensity (Hudson et al., 2007; Henderson et al., 2009; Lee et al., 2015).

3 HPLC-SEC

HPLC-SEC is a method based on separating macromolecules according to their size. The separation column is filled with porous beads with well defined pore size, and the macromolecules penetrate these pores to a smaller or greater extent, depending on their size/hydrodynamic radius. Originating in the 1950s, this technique developed in strides with improved column materials from soft gel to sub-2-μm particles, diversification of mobile phases, and introduction of a great variety of detector systems (Silberring et al., 2004; Bouvier & Koza, 2014).

Ideally, SEC is an entropy-governed equilibrium process, whereby the separation is controlled by the different extent of permeation of different macromolecules into the gel pores (Striegel, 2004). SEC analysis results in a chromatogram ideally comprised of well separated Gaussian curve-shaped peaks, each representing a single macromolecule or a mixture of macromolecules, eluted in the order of decreasing size (and correspondingly decreasing molar masses). The position of the maximum, the width of the peak, and the peak height (PH)/peak area (PA) contain the information generally sought for in analysis (Figure 1). The peak maximum retention time (Rt) can be used to assess the molar mass of a single molecule. For that, the column is calibrated with a series of macromolecules with narrow dispersity used as SEC calibration standards to find the linear relationship "logMW = f (Rt)" (Vander Heyden et al., 2002; Silberring et al., 2005). For a polydisperse sample (mixture of similar macromolecules with different molar masses eluted under the same peak), MWDs—such as number-average MWD: M_n (Equation 1) or weight-average MWD: M_w (Equation 2)—can be calculated by numerical deconvolution of the peak (Fig. 1) (Striegel, 2004; Janca, 2005). For samples that give chromatograms with well separated peaks, one molecular weight corresponding to the Rt of peak maxima is calculated (MWp) (Peuravuori &Pihlaja, 1997):

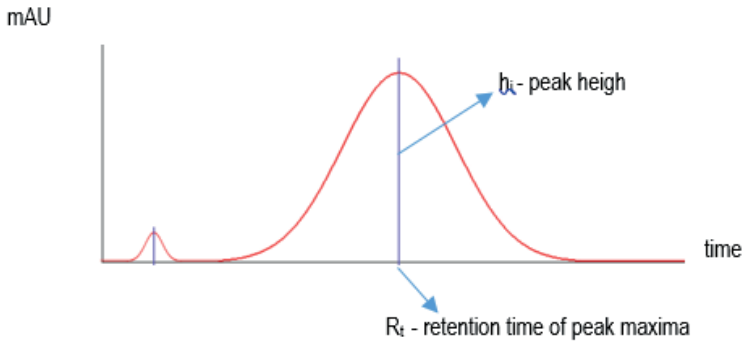


Figure 1. Peaks of a chromatogram

$$M_n = \frac{\Sigma M_i h_i}{\Sigma h_i} \quad \text{(Eq. 1)}$$

$$M_w = \frac{\Sigma M_i^2 h_i}{\Sigma M_i h_i} \quad \text{(Eq. 2),}$$

where M_n is the number-average MWD and M_w is the weight-average MWD of the sample; M_i is the MW calculated at Rt "i", and h_i is the peak height at Rt "i."

Frequently used compounds in SEC calibration are polystyrene standards of narrow MWD, polycyclic aromatic hydrocarbon standards, polysaccharides of known MW, and proteins of known MW. The standards are chosen based on the nature of the macromolecules to be analyzed (Ricker & Sandoval, 1996; Irvine, 1997; Silberring et al., 2005; Liu et al., 2006; Gomez-Ordonez et al., 2012). In calibration, the void volume (Rt of the largest calibration molecule that is not retained at all within the column) and the permeation volume (Rt of the smallest calibration molecule that completely penetrates the pores) are determined, and the Rt of the analyte must reside between these values.

In spite of its technical advances, SEC is never truly ideal, because secondary interactions (electrostatic or hydrophobic) between stationary phase and analyte molecules cannot be fully eliminated (Ricker & Sandoval, 1996; Irvine, 1997; Pujar & Zydney, 1998; Specht & Frimmel, 2000; Janos, 2003; Bouvier & Koza, 2014). To optimize SEC means, in fact, to minimize these secondary interactions. When the analyte and stationary phase have opposite charges, ionic adsorption occurs that leads to increased Rt, deformation of the peak (tailing), and underestimation of MW.

Ion repulsion occurs when the analyte and stationary phase bear the same charge, causing decreased Rt and overestimation of MW. Ionic interactions can be minimized by increasing the ionic strength (IS) and adjusting the pH of the eluent (Irvine, 1997; Pujar & Zydney, 1998). However, too high eluent IS strengthens the hydrophobic attraction between analyte and column material, which in turn causes peak tailing and increased Rt. Hydrophobic attraction can be diminished by adding an organic eluent (e.g., acetonitrile, methanol) to promote the elution of the hydrophobic analyte. (Ricker & Sandoval, 1996; Irvine, 1997; Pujar & Zydney, 1998; Bouvier & Koza, 2014). Suitable eluent pH is usually ensured by using a buffer solution of near neutral pH (Kawasaki et al., 2011; Bhatia et al., 2013; Wagner et al., 2016). In addition, operational parameters, such as injection volume and eluent flow rate, must be properly adjusted for good quality separation (Ricker & Sandoval, 1996). Recently, SEC optimization has been tailored to a specific analyte that includes minimum experimental runs based on computer simulation used to find the best separation conditions (Duarte & Duarte, 2010). Peak broadening and decreased Rt may appear when the macromolecules remain adsorbed to the stationary phase, thus reducing the separation pores of the column; hence, the columns must be regularly cleaned and calibrated (Simenkova & Berek, 2005).

SEC has been applied mainly to determine the MWD of synthetic polymers and biomolecules, such as proteins, peptides, enzymes, DNA, carbohydrates, and lipids, in industry and research (Bouvier & Koza, 2014), but it has also been used to purify peptides and study interactions among peptide molecules (Irvine, 1997). In environmental analysis, SEC is used to characterize DOM.

The column materials used in SEC today are porous silica-based material with a modified surface to decrease ionic interaction with proteins, porous hybrid organic/inorganic particles containing hybrid silanols on the surface, semi-rigid polymer packing based on crosslinking of polystyrene/divinylbenzene, dextrane cross linked with agarose, and monolith material (Silberring et al. 2004; Bauvier & Koza, 2014). The eluents used are aqueous eluents with ionic materials dissolved (phosphate salts, phosphoric acid, ammonium nitrate, sodium nitrate, sodium acetate, acetic acid, formic acid) and organic liquid (acetonitrile, tetrahydrofuran), or their mixture (Silberring et al., 2004; Liu et al., 2006; Gomez-Ordonez et al., 2012).

3.1 Use of HPLC-SEC in DOM analysis

Applications of SEC in aquatic DOM analysis started in the 1980s. TSK-silicagel-based columns were the frequently used column types, though polymer-based columns were used as well (Hongve et al., 1996; Her et al., 2002 Hoque et al.,2003; Janos & Zatrepalkova, 2007). To separate DOM, some studies applied columns, such as the Spherisorb (Lombardi & Jardim, 1998), Ultrahydrogel 120 aqueous SEC (Varga et al., 2000), Resin HW 50S and HW 55S (Lankes et al., 2009), and HMW Superdex 200 10/300 GL and LMW Agilent Bio SEC 100A (Bhatia et al., 2013). Comparative studies showed slightly better separation of humic matter with a silicagel-based column (Hongve et al., 1996; Conte & Piccolo, 1998). The preferred eluent for TSK-silicagel-based columns was low IS (up to 0.05M) phosphate buffer at about neutral pH (Chin et al.,1994; Peuravuori & Pihlaja, 1997; Pelkani et al., 1999; Zhou et al., 2000; Alberts et al., 2002; Imai et al., 2002; Hoque et al.,2003; Wu et al., 2003; Lankes et al., 2009; Kawasaki et al., 2011; Bhatia et al., 2013; Wagner et al., 2016) or a low IS neutral salt solution, such as $NaNO_3$, Na_2SO_4, and NaCl, buffered to near neutral with phosphate (Hongve et al., 1996; Her et al., 2002; Her et al., 2003; Her et al., 2004; Hur et al., 2006; Wu et al., 2007a). Another preferred eluent was low concentration Na-acetate, because in the separation of whole water sample DOM it provided a resolution better than phosphate buffer (Vartiainen et al., 1987; Peuravuori & Pihlaja, 1997; Vuorio et al., 1998; Matilainen et al., 2002; Myllykangas et al., 2002). Other eluents used in SEC have been low IS $NaNO_3$ (Conte & Piccolo, 1998) and NaClO4 (Egeberg et al., 1998), KCl combined with acetonitril (Lombardi & Jardim, 1998), Na-azide (Peuravuori & Pihlaja, 1997), and Na-tetraborate of pH 9.2 (Varga et al., 2000; Janos & Zatrepalkova, 2007).

In SEC analysis, DOM is detected mainly using UV, which, combined with online FLU and/or online DOC detection, becomes a powerful tool to characterize aquatic organic matter. Table 3 shows online detections coupled with HPLC-SEC used in DOM studies. UV detects mostly fulvic and humic fractions, whereas FLU spectroscopy is useful in detecting protein-type fractions (Amy & Her, 2004). The preferred UV absorbance wavelengths to detect DOM are 254 nm or 280 nm (Table 3). Detection wavelength is important in MWD calculations, because peak maxima shift toward higher MWs as the detection wavelength increases (Zhou et al., 2000). At a lower detection wavelength, inorganic compounds with conjugated double bonds (e.g., nitrate ion absorbing strongly at 224 nm) may interfere with detection (Ferree & Shannon, 2001). Additional online FLU or DOC detection allows

Table 3. Online detectors coupled with HPLC-SEC

Detection in HPLC-SEC	Detection	Sample type	Results	Interpretation	References
SEC –UV and FLU	254 nm + excitation spectra with fixed emission at 560 nm + emission spectra with fixed excitation at 350 nm and 450 nm + SFS with Δλ= 27 nm	solid phase extracted marine sample and reference fulvic acid	UV chromatograms, excitation spectra, emission spectra, synchronous FLU spectra	• extensive qualitative characterization of marine DOM	Lombardi & Jardim, 1998
	254 nm + EEMS	UF-fractionated surface water	UV chromatograms, 2D EEMS spectra, maxima ex/em: 335/440 nm, 225/427 nm	• extensive qualitative characterization of DOM	Alberts et al., 2002
SEC-UV-FLU and additional FLU	210 nm, 280 nm, online ex/em 210/280 nm, 345/443 nm + EEMS	extracellular polymers from biological aerobic and anaerobic wastewater treatment units	UV, FLU chromatograms, 2D EEMS spectra, maxima at 221/350 nm protein-like and 345/443 nm 335/458 nm humic-like	• extensive qualitative characterization of extracellular polymers • difference between aerobic and anaerobic extracellular polymers	Bhatia et al., 2013

SEC-UV-FLU-DOC and additional FLU	254 nm, online ex/em 260/310 nm and 330/450 nm, online DOC detection, DOC normalized emission spectra with fixed excitation at 330 nm and 450 nm	hydrolyzed XAD-fractions of surface waters	DOC-normalized emission spectra	• extensive qualitative characterization of SEC fractions • effects of hydrolysis on fractions • differences between surface water and wastewater effluents DOM • identification of products of hydrolysis	Kumke et al., 2001
	210 nm, 254 nm, online ex/em 278/353 nm and 337/423 nm, online DOC detection + EEMS	Reference humic and fulvic acid, dextran and bovine serum albumin BSA, groundwater, surface water, secondary wastewater effluent	UV-, FLU-, DOC-, SUVA- , specific FLU chromatograms FLU detection selected based on EEMS maxima	• extensive qualitative characterization of SEC fractions as protein-type, fulvic-type, polysaccharide-type fractions • differences between surface water and wastewater effluents DOM • EEMS used to choose FLU ex/em detection wavelengths	Her et al., 2003
SEC-UV-FLU-DOC and additional FLU + UV	254 nm online ex/em 278/353 nm online DOC detection + EEMS + UV-scan	algal organic matter	UV-, FLU-, DOC-, SUVA- , specific FLU chromatograms 2D EEMS	• extensive qualitative characterization of algogenic organic matter • DOM removal by nanofiltration and membrane fouling studied • EEMS and UV scan used to identify components of algogenic organic matter	Her et al., 2004

comprehensive characterization of DOM and provides simultaneously additional chromatograms that describe the aromatic (UV), fulvic-type (FLU), protein-type (FLU), and polysaccharide-type (DOC) character of the fractions or aromaticity (SUVA calculated as UV/DOC) or specific FLU (SF calculated as FLU/DOC) (Table 3).

Table 4 shows a collection from the literature of HPLC-SEC-UV, HPLC-SEC-UV-FLU, or HPLC-SEC-UV-FLU-DOC online detection coupled with complementary analysis, such as EEMS, FLU emission spectroscopy, or UV scan. These additional analyses may help choose the suitable online detection wavelengths or further identify particular components of fractions or molecules produced by hydrolysis of DOM (Table 4).

In most studies, HPLC-SEC was used to determine the MWDs of pre-fractionated DOM or those of SEC-fractionated DOM by calibrating the column with calibration standards. Permeation and void volumes were determined with acetone (58 Da) and Blue Dextrane (1 000 kDa). Narrow polystyrene sulfonates (1430 – 34 700 Da) are the common calibration standards (Chin et al., 1994; Hongve et al., 1996; Peuravuori & Pihlaja, 1997; Egeberg et al., 1998; Pelkani et al., 1999; Zhou et al., 2000; Her et al., 2002; Imai et al., 2002; Alberts et al., 2002; Wu et al., 2003; Hur et al., 2006; Wu et al., 2007a; Bahtia et al., 2013). Globular proteins (Vartiainen et al., 1987; Peuravuori & Pihlaja, 1997; Janos & Zatrepalkova, 2007; Bhatia et al., 2013), polyethylene-glycols (200-10000 Da) (Peuravuori & Pihlaja, 1997; Her et al., 2002; Her et al., 2003; Her et al., 2004; Lankes et al., 2009), and polysaccharides (Conte & Piccolo, 1998) were also used to calibrate the SEC column. Eluted fractions are classified as high molecular weight (HMW), intermediate molecular weight (IMW), and low molecular weight (LMW) fractions with distinct characteristics. High molecular weight fractions (HMW and IMW) have usually high aromaticity (Chin et al., 1994; Peuravuori & Pihlaja, 1997; Alberts et al., 2002, Her et al., 2003), but polysaccharide-type HMW fractions are not aromatic and cannot be detected with UV or FLU (Wu et al., 2003; Her et al., 2003), whereas one HMW fraction of lake water DOM showed low UV absorbance of probably microbial origin (Kawasaki et al., 2011). Standard DOM showed five fractions characterized as humic substances (16 000 Da), fulvic acids (11 000Da), IMW humic substances (6 000-10000 Da), LMW acids (5 000Da), and LMW neutrals (proteins, amino acids) (3000 Da) (Yan et al., 2012).

Table 4. Complementary FLU analysis of HPLC-SEC-UV

Detection in HPLC-SEC	Wavelength	Sample type	Results	Interpretation	Reference
UV	215 nm	reference humic and fulvic acid, whole surface water	UV chromatograms	• MWDs • peak maxima shifts to higher MWs as detection wavelength increases	Zhou et al., 2000
	224 nm	UF-fractionated surface water, commercial and reference humic and fulvic acid, pre-concentrated municipal raw wastewater and effluents	UV chromatograms	• MWDs • qualitative and quantitative characterization of WW and WWE • SEC fractions removal • column-analyte secondary interaction assessment	Crozes et al., 1996; Pelkani et al., 1999; Hur et al., 2006
	230 nm	reference humic and fulvic acid, whole surface water	UV chromatograms	• MWDs • peak maxima shifts to higher MWs as detection wavelength increases	Zhou et al., 2000
	240 nm	XAD-fractionated surface water- and groundwater	UV chromatograms	• MWDs	Hoque et al.,2003
	254 nm	commercial and reference humic and fulvic acids, XAD- or UF- fractionated and whole water samples of surface waters, artificially recharged groundwater, samples from drinking water treatment steps, drinking water, pre-concentrated municipal raw wastewater and effluents	UV chromatograms	• MWDs • qualitative and quantitative characterization of DOM • SEC fractions removal • column-analyte secondary interaction assessment	Vartiainen et al., 1987; Crozes et al., 1996; Peuravuori & Pihlaja, 1997; Conte & Piccolo, 1998; Vuorio et al., 1998; Egeberg et al., 1998; Zhou et al., 2000; Myllykangas et al., 2002; Hur et al., 2006; Matilainen et al., 2002

	260 nm	UF-fractionated surface water, whole surface water, XAD-fractionated municipal wastewater treatment plant and onsite wastewater effluent.	UV chromatograms	• MWDs • column-analyte secondary interaction assessment • effects of wastewater treatment steps on DOM fractions	Pelkani et al., 1999; Imai et al., 2002
	280 nm	UF-fractionated surface water, commercial and reference humic and fulvic acid, whole surface water	UV chromatograms	• MWDs • column-analyte secondary interaction assessment	Pelkani et al., 1999; Zhou et al., 2000;
	350 nm	reference humic and fulvic acid, whole surface water	UV chromatograms	• MWDs • peak maxima shifts to higher MWs as detection wavelength increases	Zhou et al., 2000;
UV+ FLU	280 nm, ex/em 238/380 nm	reference humic and fulvic acids	UV, FLU chromatograms	• column-analyte secondary interactions	Varga et al., 2000
	250 nm, ex/em 350/450 nm, ex/em 450/530 nm, EEMS	reference humic and fulvic acid, whole surface water, XAD-fractionated surface water	UV, FLU chromatograms, ex/em wavelengths of FLU maxima, 3D-EEMS of SEC fractions	• FLU profile of SEC fractions • qualitative characterization of DOM fractions • HMW not fluorescent, secondary interaction	Wu et al., 2003; Wu et al., 2007a
	210 nm, 280 nm, ex/em 210/280 nm, ex/em 345/443 nm	extracellular polymers from biological aerobic and anaerobic wastewater treatment units	UV, FLU chromatograms	• MWD of UV chromophores and FLU fluorophores • differentiation between protein-type and humic-type extracellular polymers • difference between aerobic and anaerobic extracellular polymers	Bhatia et al., 2013
UV+ DOC	254 nm, 280 nm, DOC detection	reference humic and fulvic acids	UV, DOC chromatograms	• column-analyte interactions	Hongve et al., 1996
	254 nm, DOC detection	reference humic and fulvic acids, albumin, sucrose	UV, DOC, SUVA chromatograms	• qualitative and quantitative SEC fraction characterization	Her et al., 2002
	254 nm, organic carbon detection	UF-fractionated surface waters	UV, OCD chromatograms	• qualitative and quantitative characterization of SEC fractions	Lankes et al, 2009
	260 nm, non- dispersive infrared TOC detection	surface water and sediment	UV, TOC chromatograms	• qualitative and quantitative characterization of SEC fractions	Kawasaki et al., 2011

UV + FLU+ DOC	254 nm, ex/em 260/310 nm and 330/450 nm, DOC detection	XAD-fractionated and hydrolyzed surface water and wastewater effluent	UV, FLU, DOC chromatograms	• extensive qualitative characterization of SEC fractions • effects of hydrolysis on fractions quality • differences between surface water and wastewater effluents DOM	Kumke et al., 2001
	210 nm, 254 nm, ex/em 278/353 nm and 337/423 nm, DOC detection	reference humic and fulvic acid, dextran and bovine serum albumin, groundwater, surface water, secondary wastewater effluent	UV, FLU, DOC, SUVA chromatograms	• extensive qualitative characterization of SEC fractions as protein-type, fulvic-type, polysaccharide-type fractions • differences between surface water and wastewater effluents DOM	Her et al., 2003
	254 nm ex/em 278/353 nm DOC detection	algal organic matter, reference humic and fulvic acids	UV-, FLU-, DOC-, SUVA- , specific FLU chromatograms	• extensive qualitative characterization of algogenic organic matter • DOM removal by nanofiltration and membrane fouling studied	Her et al., 2004
	scan at 210-450 nm, online emission spectra, OCD (organic carbon) detection	reference humic and fulvic acids, phenylalanine, tryptophan, tyrosine, surface water, drinking water, samples from drinking water treatment steps	3D UV-chromatograms, emission intensities at emission wavelengths 260-450 nm as a function of retention time, chromatograms	• extensive qualitative characterization of DOM • validation of a model used for quantitative and qualitative DOM characterization from EEMS	Wagner et al., 2016

Fraction characteristics depend mostly on the type of water. Surface water DOM had similar UV absorbance and FLU chromophores, regardless of geographic location and climate (Alberts et al., 2002). LMW fractions were mostly aliphatic in groundwater but mostly protein-type in surface water (Her et al., 2003). Extracted soil fulvic acids had higher FLU than marine DOM (Lombardi & Jardim, 1998).

In WWEs, the HMW fraction, mostly composed of extracellular polymers (Crozes et al., 1996), was of predominantly polysaccharide- and protein-type character (Her et al., 2003). In another study, WWE fractions showed mostly low MWDs, and the onsite wastewater effluent, because of its complex composition, had broader MWD plus extra LMW fractions than the municipal effluent (Imai et al., 2002). Municipal wastewater effluent showed no significant changes in its chromatograms after it was hydrolyzed under basic conditions into relatively stable and simple components (Kumke et al., 2001).

PA or PH can be used to quantify compounds. For humic matter, PA and PH were proportional to mass concentration (Janos, 2003). In general, the sum of peak heights (SPH) or the sum of peak areas (SPA) correlated with conventional DOM indicators, such as color, TOC, DOC, and COD (Vartiainen et al., 1987; Myllykangas et al., 2002; Vuorio et al., 1998; Matilainen at al., 2002).

In monitoring water and wastewater treatment, it is useful to comprehensively characterize DOM by HPLC-SEC with multiple detection, because it can show quantitative and qualitative (humic and/or microbial) DOM removal throughout the treatment chain in addition to being capable of identifying the DOM source (terrestrial allochtonous versus algal autochtonous) in surface waters (Amy & Her, 2004). HPLC-SEC studies on producing drinking water from lake water showed that the higher the weight of the fractions, the more efficient their removal in the process. HMW was removed 80-100%; IMW up to 66 %, and LMW about 33% (Vartiainen et al., 1987; Vuorio et al., 1998; Matilainen et al., 2002). Ozonation and granular activated carbon filtration improved IMW removal, and ozonation increased the LMW fraction (Vuorio et al., 1998). Myllykangas et al., (2002) discovered a similar trend in artificial recharge of lake water, where the HMW fraction was removed best. In their study, advanced oxidation of groundwater DOM resulted in small compounds identified as formate, acetate, propionate, pyruvate, oxalate, and citrate. Raw water and permeate were characterized by HPLC-SEC multiple detection in a surface water ultrafiltration (UF) unit, showing efficient HMW and aromaticity

(SUVA) reduction (Her et al., 2002). By UV and TOC detection, Crozes et al.(1996) were able to characterize DOM removal in activated sludge-treated wastewater and activated sludge + anaerobically-treated wastewater at TOC removal efficiencies of 59 and 46%, respectively, whereas the HMW fraction was better removed in the second treatment (activated sludge + anaerobic). Another study on advanced treatment of municipal effluent found that ozonation affected MWD by increasing Rt, while parallel UF combined with activated carbon filtration resulted in complete removal of HMW (Imai et al., 2002).

3.2 Sample pretreatment

Before HPLC-SEC, water samples are filtered through a 0.45μm filter, and if not treated before filtration, they are called "whole water samples." To further separate and pre-concentrate DOM, samples are often isolated and fractionated prior to SEC analysis. Surface and ground water samples, wastewater effluents, soil extracts, and reference humic matter were fractionated in many studies by XAD (Vartiainen at al., 1987; Chin et al., 1994; Peuravuori & Pihlaja, 1997; Kumke et al., 2001; Alberts et al., 2002; Janos, 2003), by UF (Egeberg et al., 1998; Pelkani et al., 1999; Wu et al., 2007a; Lankes et al., 2009), or by UF + XAD combined (Alberts et al., 2002) before they were run through a SEC column. As mentioned earlier, the laborious XAD fractionation results in three (humic acids, fulvic acids, HPI-A) or more (HPO-A, HPO-N, HPI-B, HPI-A, HPI-N, or their sub-fractions) fractions. UF separates DOM into five fractions according to MW: <500 Da, 500-3000 Da, 3000-10000 Da, 10000-30000 Da, and >300000 Da.

Despite their advantages, isolation, sample pre-concentration, and fractionation have several drawbacks. In XAD fractionation, samples are subjected to extreme pH conditions, which affect the size of the molecules by changing their tertiary structures and, further, the structures of fluorophores by causing dissociation of carboxylic and phenolic groups (Hautala et al., 1999; Zsolnay, 2003). UF is influenced by pH, acidic samples having the worst recovery (Gjessing et al., 1998). Another drawback of pre-concentration is that in highly concentrated samples DOM molecules tend to associate and cause a shift to higher MWs (Zsolnay, 2003). Even during simple filtration, organic matter can be released by cavitation (Zsolnay, 2003). Therefore, sample pre-treatment should be avoided/minimized, for otherwise results will be biased and should be interpreted with caution.

4 GAP IN THE KNOWLEDGE

Despite numerous studies of DOM by HPLC-SEC involving UV/VIS and FLU spectroscopy, the method harbors further possibilities for exploration. Spatial and temporal data on a DOM profile along a catchment would help further understand the transformations in organic matter in an aqueous environment. HPLC-SEC-UV-FLU could help describe the variation in DOM fractions along the area and provide useful information for a water treatment plant about the DOM that needs to be removed. There are no systematic studies on well water DOM from sparsely populated agricultural areas. These wells can be affected by onsite sanitation and farming activities. Studies are also unavailable on septic tanks and onsite WWE quality, though, reportedly, onsite sanitation can negatively affect the quality of nearby well water and water receiving bodies. HPLC-SEC-UV-FLU could provide valuable information on WWE and well water quality and could even help trace WWE or surface water in wells. Furthermore, HPLC-SEC provides information not only for qualitative but also for quantitative DOM characterization of onsite WWEs, which lacks systematic study at present. Chromatographic data provided by the method could be exploited to replace the conventional indicators, such as BOD-7, COD, TN, or DOC, whose determination is time consuming or laborious.

5 OBJECTIVES

This study sought to evaluate the possibilities of HPLC-SEC combined with UV and FLU detection to analyze different types of water samples not subjected to any pretreatment except filtration through a 0.45μm filter. The aim was to test the outcomes of SEC analyses as surrogates for different types of conventional water quality indicators or as pollution indicators. The focus was to fill the gap in the knowledge on HPLC-SEC applicability for rapid characterization of catchment DOM, well water, and septic tank effluent quality.

The objectives were achieved by

- determining DOM characteristics and DOM seasonal variation along a typical boreal catchment and in drinking water produced from lake water in the catchment by conventional indicators and HPLC-SEC

- assessing the general quality of well waters and the anthropogenic influence on well waters from Finnish rural areas based on chromatographic data

- developing an optimal HPLC-SEC method suitable for complex onsite analysis of wastewater effluent

- assessing the general quality of and characterize onsite wastewater effluents by the developed method

- finding reliable conventional organic matter indicator surrogates from chromatographic data.

6 MATERIALS AND METHODS

6.1 Sites, samples

In this study, we analyzed the surface waters of a catchment (drain- and lake waters), well waters, and onsite septic effluents from four locations and a secondary wastewater effluent (Table 5). The studied catchment covered an area of 117 km^2 in Joutsijärvi and Tuurujärvi in southwestern Finland, mostly forest and mire with small (3.4%) agriculture fields. Tuurujärvi is the source of drinking water for the city of Pori (about 85,000 population), and the water is treated by coagulation, flotation, sand filtration, artificial groundwater recharge, and disinfection before being distributed as drinking water. Of the drainage area, 84% belongs to two large drains, one (Jylhäoja) comprising most of the agricultural activities of the region, the other (Ahmausoja) collecting water from a drained mire and mixed coniferous and deciduous forest area. Over the past decades, the lakes have shown increasing color values. The site is described in detail in Paper I.

Well water samples were collected from private wells in four different, sparsely populated regions in western, eastern, and southern Finland. The sampling sites are affected by agricultural activities. Wastewater effluents were taken from small-scale, onsite wastewater of single households in western and southern Finland. A secondary effluent sample was collected at the municipal wastewater treatment plant in Tampere, Finland.

6.2 Sampling, analysis

Sampling characteristics, sample types, and numbers are given in Table 5, and the conventional quality indicators and the methods of measurement are given in Table 6. For HPLC-SEC measurements, pre-filtered whole water samples were fractionated with a Hewlett-Packard HPLC 1100 system with a TSKgel G3000SW 7.5 mm x 30cm column (3 columns used during the studies), and detected with a UV/VIS HP 1100 Series Diode Array Detector in tandem with an HP 1100 Series

FLU Detector (Fig. 2). The selected detection wavelengths, based on previous studies, were 224 nm and 254 nm for the UV/VIS detector and ex/em 270/310 (tyrosine-like) and ex/em 270 nm/355 nm (tryptophan-like) for the FLU detector (Ahmad et al., 1995; Baker, 2002). Because we employed no online DOC detector, we used an Na-acetate solution as eluent, because it gave a higher resolution for NOM fractionation than a phosphate buffer (Peuravuori & Pihlaja, 1997).

The column was calibrated with sodium salts of polystyrene-sulfonates (PSS) of the following MW: 210 Da, 4300 Da (FLUKA, Germany), 6800 Da, 13000 Da (FLUKA, Switzerland), and 2200 Da (Polymer Standard Service, Germany). In experiments to optimize the method for complex wastewater effluent analysis, globular proteins were used additionally to calibrate the column: tyrosine (MW = 181Da), tryptophan (MW = 204 Da, Sigma-Aldrich, Japan), β-Lactoglobuline (from bovine milk, MW = 18000 Da, Sigma, USA), and bovine serum albumin (MW = 66000 Da, Sigma, USA), as well as vitamin B12 (MW = 1400 Da, Sigma-Aldrich, China). Void volume and permeation volume were determined with Blue Dextrane (1000000Da) and acetone (58 Da). The void volume was 5 mL ± 0.01 mL and the permeation volume 12 ml ± 0.1 mL. Calibration equations, calculated by using the Rts of the peak maximum of standards, were used to assess the MWp of eluted DOM fractions (Table 12).

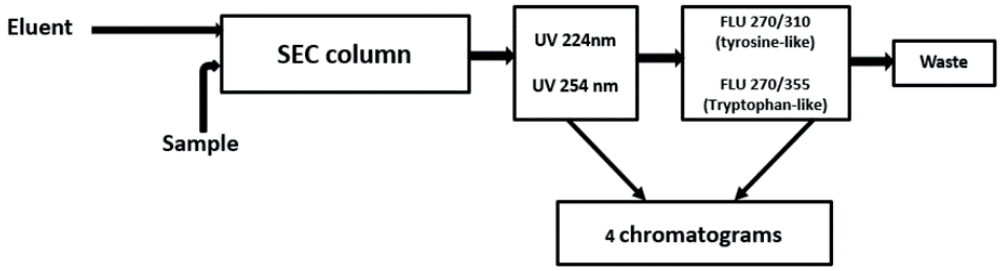


Figure 2. Schematics of the HPLC-SEC-UV-FLU system

In the experiments to optimize the method (III), we studied the effect of eluent IS, sample injection volume, and eluent pH on the separation of wastewater effluent

Table 5. Description of the samples used in this study

Sample type		Number	Sampling place	Season	Pretreatment
Well water	Shallow well, dug or spring depth 0.8-10m	193	tap	All seasons, sampled once	Filtered through a 0.45-µm filter Analyzed within 5 days from sampling or frozen until analysis
	Deep well, borehole depth 20-133 m	74	tap		
Onsite wastewater effluent	Blackwater effluent	54	from the supernatant of last (second or third) compartment of septic tank or from distribution well after septic tank or from manhole after filtration field		
	Greywater effluent	15			
Municipal wastewater effluent		1	effluent release point		
Surface water	Drain	2 (x 6)	20 cm from surface	COD monitored for 6 years, all seasons HPLC-SEC monitored for 1.5 years, all seasons	
	Lake	2 (x 6)	50-100 cm from surface		
	Drinking water	1 (x 6)	release to distribution system		

Table 6. Conventional quality indicators measured in this study

Sample type	pH	Cond. mS/cm	DOC mg/L	COD-Mn mg/L	BOD-7 mg/L	Total-N mg/L	$NO_{2,3}$-N mg/L	TON mg/L	NO_3^- mg/L	TDP mg/L
Well waters	x	x	x	x					x	
Surface waters	x	x	x	x		x	x	x		
Wastewater effluents	x	x	x	x	x	x				x

DOC-dissolved organic carbon measured with a SHIMADZU TOC-5000 analyzer
COD-chemical oxygen demand measured by permanganate titration
BOD-7 – 7-day biochemical oxygen demand, measured with Oxitop
Total-N –total nitrogen measured by the Kjeldahl method

$NO_{2,3}$-N – nitrite- and nitrate nitrogen, measured spectrophotometrically
NO_3^- - nitrate, measured by ion chromatography
TON-total organic nitrogen, calculated as (Total-N) – ($NO_{2,3}$-N)
TDP – total dissolved phosphorous, measured spectrophotometrically

DOM. For each eluent condition, three calibration equations were determined: with PSS standards only (using acetone to determine the permeation volume), with protein standards only (using tryptophan to determine the permeation volume), and with all standards (PSS + proteins).

After peaks were visually identified, chromatograms were integrated with the "Agilent ChemStation for LC systems." PH, SPH, PA, and SPA constituted the chromatographic information to quantitatively characterize total organic matter and its fractions. Table 7 shows the conditions used in the HPLC-SEC experiments.

Table 7. Conditions used in HPLC-SEC-UV-FLU and the quantitative information gained

Paper	**Eluent Na-Acetate concentration, M**	**Eluent Na-Acetate pH**	**Sample injection volume, µL**	**Quantitative chromatographic information gained**
Well waters, surface waters, wastewater effluents (II)	0.01	7.1	30	PH, SPH
Surface waters, drinking water (I)	0.01	7.1	30	PH, SPH
Optimization experiments for onsite wastewater effluent analysis (III)	0.01 0.02 0.03 0.05 0.1 0.2 0.5 1 — pH = 7; injection volume 40 µL	5.5 6.2 6.8 7 7.2 7.7 8.2 — eluent concentration 0.02 M; injection volume 40 µL	10 20 30 40 — pH = 7; eluent concentration 0.02 M	PA, SPA
Onsite wastewater effluents (IV)	0.02	7	20	PA, SPA

PA-peak area, SPA-sum of peak area (mAU*min)
PH-peak height, SPH-sum of peak heights (mAU)

6.3 Assessment of optimum HPLC-SEC conditions in complex wastewater effluent analysis

The quality of separation was assessed by calculating the value of the chromatographic response function (CRF) and global resolution from the expression CFR (Eq. 3), proposed by Duarte & Duarte (2010):

$$\mathrm{CRF} = \sum_{l-1}^{N-1} \theta_{s,l} + N - \left(\frac{t_L - t_0}{t_L}\right) \qquad (\mathrm{Eq.}\ 3)$$

where $\sum_{l-1}^{N-1} \theta_{s,l}$ is the global resolution; $\theta_{s,l}$ is the estimate of the resolution between two consecutive peaks; N is the number of peaks; t_L is the Rt of the last peak eluted within the SEC calibration range; t_0 is the Rt of the first peak eluted in SEC calibration.

The term $\theta_{s,l}$ between two adjacent peaks was calculated with Eq.4:

$$\theta_{s,l} = 1 - \frac{H_v\,(t_l - t_s)}{(t_v - t_s)|H_l - H_s| + Hs(t_l - t_s)} \qquad (\mathrm{Eq.}\ 4),$$

where H_v is the height of the valley, and t_v is the Rt of the valley between two peaks; H_s and t_s are the height and Rt of the first peak, respectively; and H_l and t_l are the height and Rt of the second peak, respectively.

To assess the effects of eluent conditions on the MWp of wastewater effluent fractions, we considered the sum of the MWp of the first four fractions after the void volume. These fractions, eluted within the calibration range, showed the highest variability in Rt and were mostly present in all samples. We calculated the sum of their MWp for clear differences, because the differences in the MWp of single fractions were not large enough.

6.4 Statistical analysis

The strength of the relationships between measured water quality indicators was assessed by statistical analysis performed by using the SigmaStat for Windows

Version 3.00 (SPSS Inc.) and the SPSS version 23 (IBM). Data was first tested for goodness of fit to a normal distribution with the one-sample Kolmogorov-Smirnov test (papers II and IV). Since none (paper II) or only 6 out of 23 (paper IV) examined indicators showed a normal distribution, the strengths of associations were evaluated with Spearman's rank correlation coefficient. A probability value of $P<0.01$ (paper II) or $P<0.01$ and $P<0.05$ (paper IV) were considered statistically significant. Linear regressions between correlating data (paper IV), calibrations (papers II, III, IV), and Pearson correlations (paper I) were done with Excel.

7 RESULTS AND DISCUSSION (PAPERS I, II, III, IV)

7.1 Experiments to optimize the analysis of wastewater effluents, interactions within the column

7.1.1 Calibration

The chromatograms were complex for BWE and effluents GWE and showed fewer fractions for aerobically treated municipal treatment plant effluent (MTPE) (Fig. 3). Typical "humic-type" fractions found in surface and well

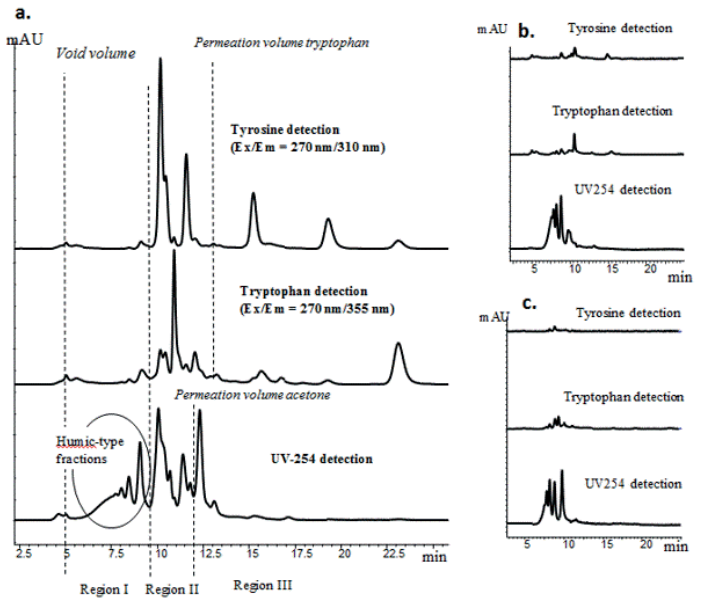


Figure 3. Chromatograms at different detections of blackwater effluents (a), greywater effluents (b), and municipal treatment plant effluent (c). Eluent: 0.02 M CH_3COONa, pH: 7.2, *mAU* (milliAmpere Units).

water samples were present in all samples. Additionally, BWE and GWE samples showed LMW fractions as detected most intensely by protein-type detection. Some of these fractions were eluted after the permeation volume. According to SEC theory**,** fractions eluted before the void volume and over the permeation volume are influenced by secondary (enthalpic) interactions; consequently**,** their MWs cannot be calculated (Irvine, 1997; Ricker & Sandoval, 1996; Pelekani et al., 1999; Sprech & Frimmel, 2000; Janos & Zatrepalkova, 2007).

Different eluent conditions led to different constants in the calibration equations (Table 8). The Rt of all calibration standards changed with changing eluent pH and concentration, except for Blue Dextrane and acetone. The longest Rt was measured at the lowest eluent pH of 5.5, which then decreased up to 6.8. At eluent pH values above 6.8, the Rt of calibration standards remained more or less stable. In addition, the peak shape of the calibration standards varied, and PA decreased in acidic eluents. This shows that, as reported before, protonated molecular forms are different in structure and lower in UV-254 absorbance and tryptophan and tyrosine FLU than deprotonated forms present at eluent pH >7 (Andersen et al., 2000; Weishaar et al., 2003; Spencer et al., 2007b; Lakemond et al., 2000; Manceva et al., 2004; Mote et al., 2010; Baker et al., 2007).

Increase in eluent IS increased the Rt of the calibration standards with a greater effect on PSS than on protein standards, a trend observed before and attributed to increased hydrophobic attraction and reduced ionic repulsion between solute and stationary phase at high eluent concentrations (Irvine, 1997; Ricker & Sandoval, 1996; Pelekani et al., 1999; Janos & Zatrepalkova, 2007; Figueruelo et al., 2004; Her et al., 2003; Hoque et al., 2003; Pujar & Zydney, 1998). Because separate calibrations with PSS and protein standards produced high R^2 values, we recommend separate PSS calibration for the UV-254 signal and protein calibration for the tyrosine and tryptophan signals. As for the nature of the interactions, R^2 values were over 0.9 for various eluent pH and IS values, indicating that size exclusion is the governing mechanism to separate calibration standards. An eluent IS of over 0.5 M strengthens the hydrophobic interactions between the column gel and the calibration standards of different MWs, causing a decrease in R^2. Eluent pH affects interactions only at an acidic (pH 5.5) value (Table 8).

Table 8. HPLC-SEC column calibration with Polystyrene-Sulfonates (PSS) and Protein standards

IS (M)	PSS			PSS + Proteins			Proteins		
	log(Mw) = a - b*Rt								
	a	b	R^2	a	b	R^2	a	b	R^2
0.01	5.383	0.3023	0.9537	5.4188	0.2623	0.7609	6.2157	0.3054	0.9009
0.02	5.6927	0.3094	0.9622	5.8285	0.2875	0.7876	6.7705	0.3445	0.9179
0.03	5.9835	0.3261	0.9519	6.1672	0.3124	0.7915	7.1934	0.3777	0.9146
0.05	6.3659	0.3484	0.933	6.6202	0.3466	0.8083	7.6071	0.4106	0.9084
0.1	6.765	0.369	0.8922	7.1477	0.385	0.8174	8.1016	0.4496	0.9013
0.2	7.0845	0.3828	0.8402	7.5777	0.4141	0.8201	8.4097	0.4725	0.8931
0.5	7.2312	0.3796	0.7606	7.8165	0.4243	0.798	8.5382	0.4768	0.8776
1	6.9901	0.3455	0.6797	7.6482	0.3975	0.7573	8.3734	0.4542	0.8578
pH	PSS			PSS + Proteins			Proteins		
	log(Mw) = a - b*Rt								
	a	b	R^2	a	b	R^2	a	b	R^2
5.5	6.2897	0.3265	0.8648	6.8257	0.3564	0.7653	8.4134	0.4782	0.9009
6.2	6.0214	0.3303	0.9622	6.2744	0.3167	0.7869	7.453	0.4035	0.9180
6.8	5.6696	0.3176	0.9616	5.723	0.2777	0.7174	7.0185	0.3688	0.9277
7	5.6927	0.3094	0.9622	5.8285	0.2875	0.7876	6.7705	0.3445	0.9179
7.2	5.6387	0.3038	0.9557	5.7809	0.2802	0.7659	6.859	0.3551	0.9201
7.7	5.6315	0.3035	0.9583	5.7689	0.2803	0.7789	6.7478	0.3471	0.9159
8.2	5.6367	0.3028	0.9539	5.8588	0.2971	0.817	6.6334	0.3447	0.9004

R^2 = regression coefficient; a and b-constants in calibration equations; Mw-molecular weight (Da); Rt-retention time (min); IS-eluent ionic strength (mol/L)

7.1.2 Molecular weights (MWp) of wastewater effluent fractions and quality of separation

The sum of the MWp of four peaks and correspondingly the Rt of these peaks varied with changes in both the IS and pH of the eluent. The MWs decreased significantly with increasing eluent IS up to 0.1M and remained nearly constant at IS > 0.1 to 0.2 M. Similarly, an increase in eluent pH up to 6.8 caused significant changes in MWs, though fewer at pH > 7. The observed variation can be explained by (1) changes in eluent conditions modifying to a greater extend the size and shape of sample macromolecules than those of the calibration standards, and (2) secondary interactions being stronger in the case of sample molecules than in the case of calibration standards. A high eluent concentration strengthens solute-column hydrophobic interactions, whereas acidic eluent pH may reduce negative charges in the stationary phase, which in turn leads to a weakening of the ionic repulsion between sample molecules and column gel with an increase in Rt (Irvine, 1997; Pelekani et al., 1999). On the other hand, variations in pH have induced changes in the structure and size of the macromolecules (Andersen et al., 2000; Weishaar et al., 2003; Spencer et al., 2007; Lakemond et al., 2000; Manceva et al., 2004; Mote et al., 2010; Baker et al., 2007).

To assess the quality of separation, we calculated a global resolution for UV-254, tryptophan, and tyrosine detections and the sum of the resolutions (SUM Resol) (Table 9). Injection volume, eluent IS, and pH all influenced the separation. For less concentrated MTPE, higher injection volumes (30 and 40 μL) and for more complex BWE, lower injection volumes (10 and 20 μL) produced better resolutions. Apparently, injection volume had no effect on GWE.

Trends were observed in resolutions varying with changes in both eluent pH and IS. The fractions influenced mostly by eluent pH were those within the calibration range, whereas those eluted after 15 min varied slightly only in Rt. Similar resolutions were calculated for an eluent pH of over 6.8, both individual and SUM Resol, except for the GWE 1 tyrosine-signal, which showed a "ghost" peak at 19 min. Smaller resolutions at low eluent pH values (5.5 and 6.2) were caused by peak compression from changes in the structures of large protein or humic molecules and from

Table 9. Global resolution of five wastewater effluent chromatograms at three different detections (UV-254 nm; Tryptophan 270/355; Tyrosine 270/310) for different injection volumes, eluent concentrations, and eluent pH values (*SUM Resol. –sum of resolutions). Greatest values in bold type.

Wastewater effluent	MTP				GWE 1				GWE 2				BWE 1				BWE 2			
Detection	254	Tryptophan	Tyrosine	SUM Resol.*	254	Tryptophan	Tyrosine	SUM Resol.*	254	Tryptophan	Tyrosine	SUM Resol.*	254	Tryptophan	Tyrosine	SUM Resol.*	254	Tryptophan	Tyrosine	SUM Resol.
Injection volume µL																				
10	3.4	2.6	0.0	6.0	6.0	***6.9***	6.2	19.1					10.5	***16.3***	***12.3***	39.0				
20	4.3	3.1	0.0	7.5	***6.9***	6.2	6.3	19.4					***10.9***	12.4	10.3	33.5				
30	***4.5***	4.1	1.8	10.3	6.4	6.3	5.3	17.9					9.9	10.6	10.8	31.4				
40	4.1	***5.1***	***1.8***	10.9	5.2	6.7	***6.7***	18.7					9.3	8.5	11.8	29.7				
Eluent concentration M																				
0.01	***6.3***	***7.7***	***3.0***	17.0	***7.4***	***7.5***	***5.1***	***20.0***	***4.8***	4.4	2.0	***11.2***	***12.6***	***15.1***	***13.6***	***41.3***	***8.7***	12.3	6.8	***27.8***
0.02	3.5	6.0	1.7	11.1	6.2	5.3	4.6	16.1	3.1	***5.1***	2.0	10.2	11.6	12.7	12.3	36.6	6.9	10.3	***6.9***	24.1
0.03	3.6	3.9	2.1	9.6	4.8	4.7	3.7	13.2	3.5	3.2	2.5	9.2	9.1	11.3	11.4	31.8	7.8	8.7	4.6	21.1
0.05	2.7	3.9	1.6	8.2	4.3	3.6	3.5	11.4	2.7	3.1	2.7	8.5	8.4	10.0	10.5	28.9	5.7	5.9	5.4	17.0
0.1	2.4	3.1	1.3	6.7	2.8	3.0	1.8	7.5	2.2	3.4	***3.0***	8.6	7.2	10.1	8.8	26.1	3.8	6.4	3.5	13.7
0.2	1.5	2.8	1.0	5.3	2.6	3.6	2.0	8.1	1.8	3.7	2.0	7.5	6.7	10.0	7.4	24.0	3.5	4.3	3.5	11.3
0.5	2.5	2.9	1.0	6.4	3.5	3.1	3.5	10.1	1.7	3.8	2.0	7.5	6.5	8.8	8.2	23.6	3.8	3.8	4.1	11.7
1	2.2	2.5	1.0	5.7	3.6	3.7	3.6	10.9	1.0	3.0	1.0	5.0	6.3	8.5	7.8	22.5	4.0	3.6	3.2	10.8
Eluent pH																				
5.5	2.3	1.8	1.1	5.1	2.7	4.9	***8.0***	15.5	4.2	3.4	2.7	10.4	8.4	11.6	9.4	29.4	5.3	7.6	5.4	18.3
6.2	3.2	2.9	1.3	7.4	4.5	7.0	4.4	15.9	4.1	4.1	2.5	10.7	9.9	12.5	9.8	32.2	5.3	10.7	8.1	24.1
6.8	***4.2***	***8.0***	1.9	***14.1***	5.2	7.7	3.9	16.8	4.2	***4.5***	2.7	11.4	10.3	***14.1***	11.5	35.9	7.3	10.3	7.5	25.1
7.2	4.2	4.3	1.8	10.3	***6.9***	7.7	4.7	19.4	***6.5***	***4.5***	***4.1***	***15.1***	***12.0***	13.8	12.7	***38.5***	***8.4***	***12.8***	***9.2***	***30.4***
7.7	3.5	3.6	***2.3***	9.4	6.3	8.2	5.7	20.2	5.8	***4.5***	2.4	12.7	10.8	12.1	12.8	35.7	7.3	11.7	7.7	26.7
8.5	3.6	4.3	1.9	9.7	5.6	***8.4***	7.2	***21.3***	5.9	4.3	2.8	13.0	10.8	12.8	***13.3***	37.0	***8.4***	12.6	7.0	28.0

increased repulsion between dissociated solute and column material, as reported before (Lakemond et al., 2000; Mote et al., 2010; Manceva et al., 2004; Hongve et al., 1996). Dependence on UV-254 absorbance pH was assessed with SPA values, which were also high at neutral or basic eluent pH in agreement with previous findings (Andersen at al., 2000; Weishaar et al., 2003; Spencer et al., 2007). The variation in tryptophan- and tyrosine-like FLU with eluent pH evinced no clear trend, which was expected in light of the highly variable effluents composition.

Changes in eluent IS caused changes in Rt, resolutions, and cromatogram PAs (Fig. 4). The lowest eluent concentration of 0.01M produced the highest resolution in the chromatograms. An increase in eluent IS diminished the governing ionic interactions and strengthened hydrophobic attractions between solute molecules and the stationary phase, causing an increase in the Rt of the peaks. Humic-type molecules (Hongve et al., 1996; Pelekani et al., 1999; Janos & Zatrepalkova, 2007; Her et al, 2003; Hoque et al., 2003), proteins and other biomolecules (Irvie, 1997; Ricker & Sandoval, 1996; Pujar & Zydney, 1998; Renard et al., 1998), simple alcohols, organic acids, and amino acids (Sprech at al., 2000) showed similar behavior. Most WWE showed a slight increase in UV-254 absorbance at an eluent pH of over 0.05 M, as previously reported (Rubia et al., 2006; Janot et al., 2010). No clear trends were observed in the variation of tryptophan-like FLU with eluent IS, whereas tyrosine-like FLU decreased clearly with increasing eluent IS (Feitelson, 1964; Teale, 1960) and possibly even with sodium ion in the eluent (Kilhoffer et al., 1981).

Based on this study, for WWE sample analysis, we recommend a low Na-acetate concentration of maximum 0.03M of neutral or slightly basic pH and an injection volume of 20 μL for complex WWE.

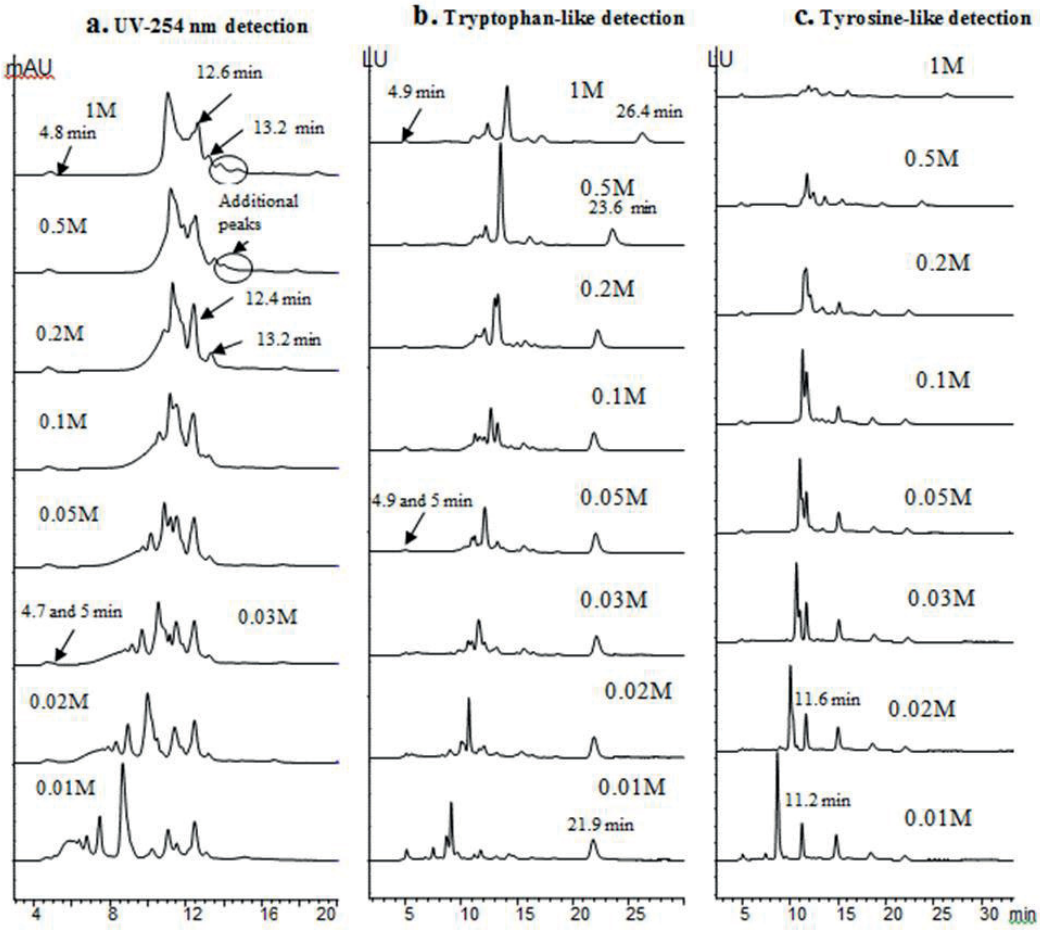


Figure 4. Chromatograms of blackwater effluent BWE1 at different eluent concentrations. Constant eluent pH = 7,injection volume 40 uL. *mAU* (milliAmpere Units), *LU* (Luminescence Units)

7.2 Characterization of surface waters, well waters, and wastewater effluents with conventional indicators

In the catchment study, the measured DOC values of lake waters (median 13.2 mg/L for Lake Joutsijärvi and 8.8 mg/L for Tuurujärvi) and drains (median 19.5 mg/L for Jylhäoja and 21.3 mg/L for Ahmausoja) are those commonly measured for lakes and drains in Finland (Kortelainen et al., 1989; Nissinen et al., 2001; Mattson et al., 2003).

Ahmausoja with its extensive mire and forest area, which increases DOC export in boreal catchments (Laudon et al., 2004; Mattson et al., 2005), showed, in fact, a higher DOC than Jylhäoja. DOC values decreased from drains to lakes with Joutsijärvi having 18-59% less DOC and raw water Tuurujärvi having 35-75% less DOC than the drains. This finding agrees with previous studies, showing that lakes function as DOC sinks via microbial degradation and sedimentation (Rantakaari et al., 2004; Mattson et al., 2005). Drinking water DOC showed 73-94% elimination in drains.

The drains' seasonal COD (6 years) and DOC (1.5 year) were at their lowest during winter frost, then increased during spring and summer, and reached their peak in late fall (Fig. 5). DOC increases because soil microbes are highly active in summer and fall, producing organic matter that leaches into the drains (Hongve et al., 1999; Scott et al., 2001).

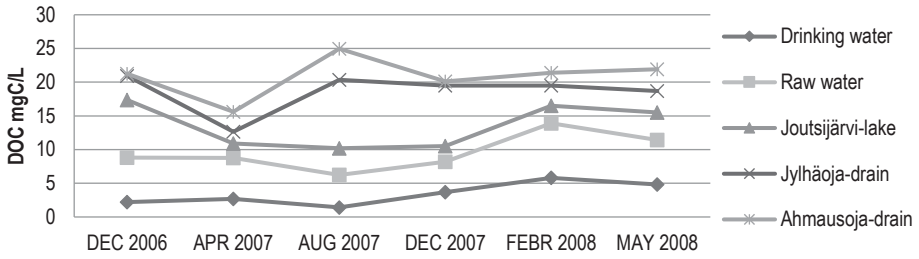


Figure 5. Seasonal variation and elimination of DOC in the catchment and water treatment plant

Unlike drains, the lakes underwent a lag in DOC and COD, showing lowest organic content in late summer and maximum in late winter (February), as reported before (Matilainen et al., 2002, Andersen et al., 2002). Decreased photo- and biodegradation of DOM following the autumn mixing in dark and cold boreal winters is likely to cause an increase in DOC (Andersen et al., 2002; Rantakaari et al., 2004; Mattson et al., 2005). Drinking water registered a DOC one magnitude lower than Tuurujärvi raw water, but showed seasonal variation in tune with that in raw water. Lowest drinking water DOC values were measured in summer 2007 (1.3 mg/L) and highest in winter and spring 2008 (5.8 and 4.8 mg/L). The sensitivity of flotation and filtration to low temperature may account for increased DOC in drinking water (Matilainen et al., 2005; Matilainen et al., 2006).

The well water survey shows the same range in DOC in deep and shallow wells (Table 10a and 10b). In Finland, the recommended DOC value for drinking water is 2 mg/L (Finnish Ministry of Social Affairs and Health, 1994); therefore, the DOC levels measured in the wells can be considered elevated, because 45 % of the shallow and 37.8% of the deep wells showed a value higher than the recommended.

Table 10a. Descriptive statistics of the quality indicators of shallow wells (n=193)

Indicator (recommended or guideline values)*	Size	Mean	Std Dev	Max	Min	Median	10 %	90 %	Over guideline (n, %)
NO_3^- (mg/L) (<50 mg/L) (>10 mg/L)**	193	14.2	24.6	219.9	0	4.9	0.1	41.6	13 (6.7 %)
DOC (mg/L) (<2 mg/L)	193	2.5	2.1	10.9	0	1.8	0.7	5.6	75 (39 %)
SPH-254 nm (mAU)	193	4.8	5.4	29.9	0.06	2.6	0.8	13.1	87 (45 %)

Table 10b. Descriptive statistics of the quality indicators of deep wells (n=74)

Indicator (recommended or guideline values)*	Size	Mean	Std Dev	Max	Min	Median	10 %	90 %	Over guideline (n, %)
NO_3^- (mg/L) (<50 mg/L) (>10 mg/L)**	74	11.8	21.3	126.4	0	0.6	0	40	5 (6.8 %)
DOC (mg/L) (<2 mg/L)	74	2.4	2.5	11.8	0.01	1.5	0.5	5.3	25 (33.8 %)
SPH-254 nm (mAU)	74	5.4	6.8	31	0	2.5	0.56	12.1	28 (37.8 %)

* according to WHO, 2004; ** increased value set according to Gelberg et al.,1999

As regards health-related quality defects, nitrate concentrations were in excess of the guideline value of 50 mg/L in similar proportions in both deep (6.8%) and shallow (6.7%) wells. Moreover, nitrate concentrations of over 10 mg/L, considered elevated

by Gelberg et al. (1999), appeared in similar proportions in deep (33.8%) and shallow wells (39%) as well. Since background nitrate levels in ground waters in Finland are less than 0.2 mg/L (Korkka-Niemi, 2001), these elevated concentrations likely resulted from anthropogenic activities.

The mean, maximum, and minimum values of the conventional indicators for onsite wastewater effluents (Table 11) are similar (TDP and BOD) or higher (TN and COD) than those in the literature for BWE (Reide Corbett et al., 2002; Jenssen et al., 2010; O'Luanaigh et al., 2012) and higher (TN and TP) for GWE (Brandes 1978). As expected, the mean GWE values for all conventional indicators were lower than the BWE values.

Table 11. Characteristics of blackwater effluents BWEs (a) and greywater effluents GWEs (b)

Parameters	a (BWE)					b (GWE)				
	Sample size	Minimum	Maximum	Mean	Std. Deviation	Sample size	Minimum	Maximum	Mean	Std. Deviation
*pH	54.0	5.6	8.8	7.2	0.6	15.0	5.9	7.9	6.8	0.5
*TDP (mg/L)	54.0	0.4	31.0	11.3	6.1	15.0	0.2	12.7	2.7	3.2
*TN (mg/L)	54.0	6.0	390.6	107.9	76.3	15.0	0.4	224.0	26.5	56.0
*BOD-7 (mg/L)	54.0	5.6	543.0	269.7	150.1	14.0	14.1	509.0	177.1	156.6
*COD (mg/L)	41.0	41.0	2853.0	661.9	498.5	11.0	35.0	980.0	389.8	286.8
*DOC (mg/L)	54.0	5.3	246.0	91.8	58.9	15.0	5.1	244.0	60.9	63.1

TDP-Total dissolved phosphorous; Tot-N-Total Kjeldahl Nitrogen; BOD-7-biochemical oxygen demand; COD-chemical oxygen demand; DOC- dissolved organic carbon. *Data published in Hyttinen (2007)

The biodegradability of GWE and BWE, assessed as BOD-7/COD, is > 0.4 (0.41 for BWE and 0.46 for GWE), indicating that the effluents will further degrade in the environment (Chamarro et al., 2001). However, the environmental loading from septic tanks depends on the number of persons using the systems and their water consumption (Hyttinen, 2007).

7.3 HPLC-SEC analysis

7.3.1 HPLC-SEC-UV224

During analysis of well water samples of high nitrate concentration, UV-224 chromatograms revealed a pronounced peak at the Rt of the LMW fraction (around 9.4 min), as yet not reported in the literature. Pure nitrate solutions (of concentration 6 - 200 mg/L) run through the column showed that the peak was indeed produced by nitrate. An early elution of nitrate ion, observed before on a Macrosphere GPC 60 column with 0.05 M ammonium acetate eluent in a cucumber sample (Mihucz et al., 2000), resulted from a secondary ionic repulsion between negatively charged nitrate ion and negatively charged silicagel column. To determine nitrate concentrations by HPLC-SEC, the column was calibrated using eight nitrate concentrations (0-200 mg/L) and obtaining R^2= 0.999 for UV-224 (Fig. 6) and R^2= 0.992 for UV-254 nm detection (not presented).

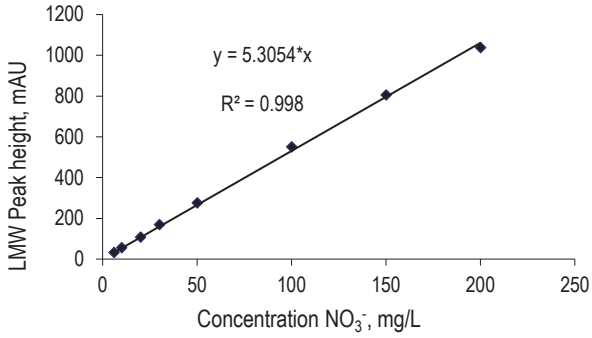


Figure 6. Calibration of a column with nitrate at UV-224 nm detection

Comparing nitrate values of 67 well water samples measured by IC and HPLC-SEC, we obtained regression equations of R^2= 0.9742 for UV-224 nm (Fig. 7) and R^2= 0.6554 for UV-254 nm detection (see Fig. 2 in Paper II). According to the regression equation, HPLC-SEC-UV224 measures slightly higher nitrate values (> 1.2 mg/L) than IC, because LMW compounds are eluted together with nitrate, which contributes to UV-224 nm absorption.

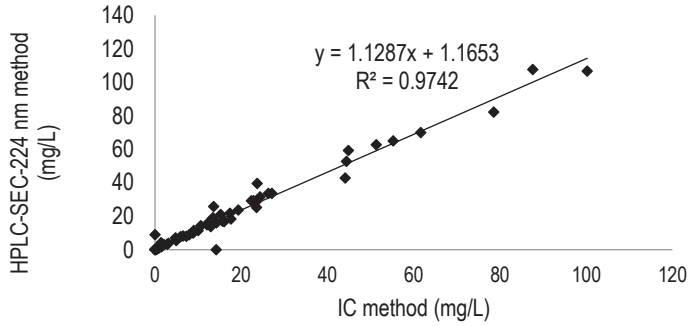


Figure 7. Nitrate measured by ion chromatography (IC) and HPLC-SEC at UV-224 nm detection

UV-224 nm can be used to distinguish between aerobically and anaerobically treated wastewater effluents (Fig. 8). The MTPE underwent an activated sludge phase, which transformed N-compounds into nitrate (113.5 mg/L measured by HPLC-SEC), BWE, and GWE, which are septic tank effluents with low nitrate peaks but many organic fractions and retarded peaks, characteristic of anaerobically digested effluent.

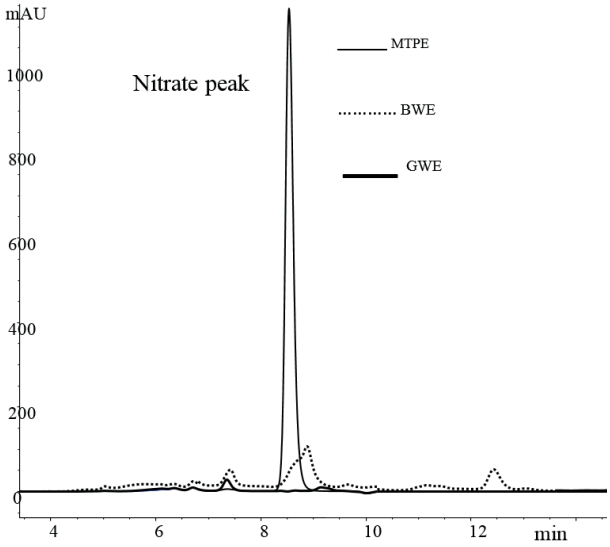


Figure 8. Chromatograms of municipal treatment plant effluent (MTPE), blackwater effluent (BWE), and greywater effluent (GWE) at UV-224 nm detection. Eluent 0.01 M Na Acetate, pH 7.0, injection volume 20 µL

7.3.2 HPLC-SEC-UV254

As seen in Figures 9 and 10, the UV-254 chromatograms of surface waters and drinking water illustrate well the elimination of DOM along the catchment and DTP and show clear differences between surface waters, WTPE, and BWE as well as well waters with different characteristics.

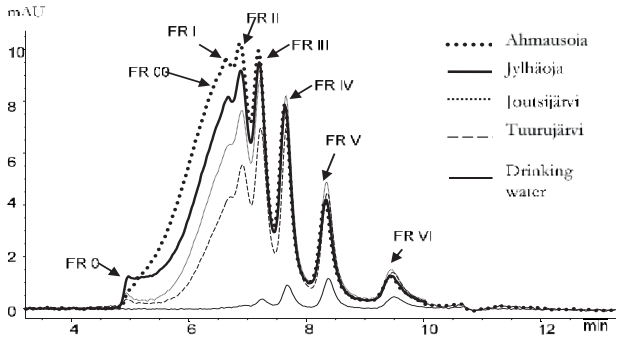


Figure 9. HPLC-SEC-UV254 chromatograms of drains, lakes, and drinking water. Eluent 0.01 M Na-Acetate

The fraction "0" is probably an association of smaller molecules and the fraction "00" a "shoulder" of fraction 1, found in drains and, except for summer samples, also in lake water samples (Fig. 10). Fractions 0, 00, I, II, and II are HMW fractions; IV and V are IMW fractions, and VI is an LMW fraction. Onsite BWE showed fractions eluted after the permeation volume (from VII on), which are affected by secondary interactions and whose MWp we cannot assess. Calculated MWp ranges (Da) for fractions eluted within the calibration range are given in Table 12.

Table 12. MWp (molecular weight corresponding to peak maxima) ranges of fractions separated by HPLC-SEC for well water and surface water samples. Eluent Na-Acetate 0.01M, pH=7.1

Calibration equations of the columns	Fractions Well waters	MWp range of the fractions (Da)
log MWp = 5.4437-0.3053*Rt	0	8 000 – 10 000
	00	3 000 – 3 800
log MWp = 5.4639 – 0.315*Rt	I	2 600 – 3 300
	II	2 200 – 3 000
log MWp = 5.4375- 0.301*Rt	III	1 900 -2 500
	IV	1 500 – 1 800
	V	900 – 1 200
	VI	380 - 470

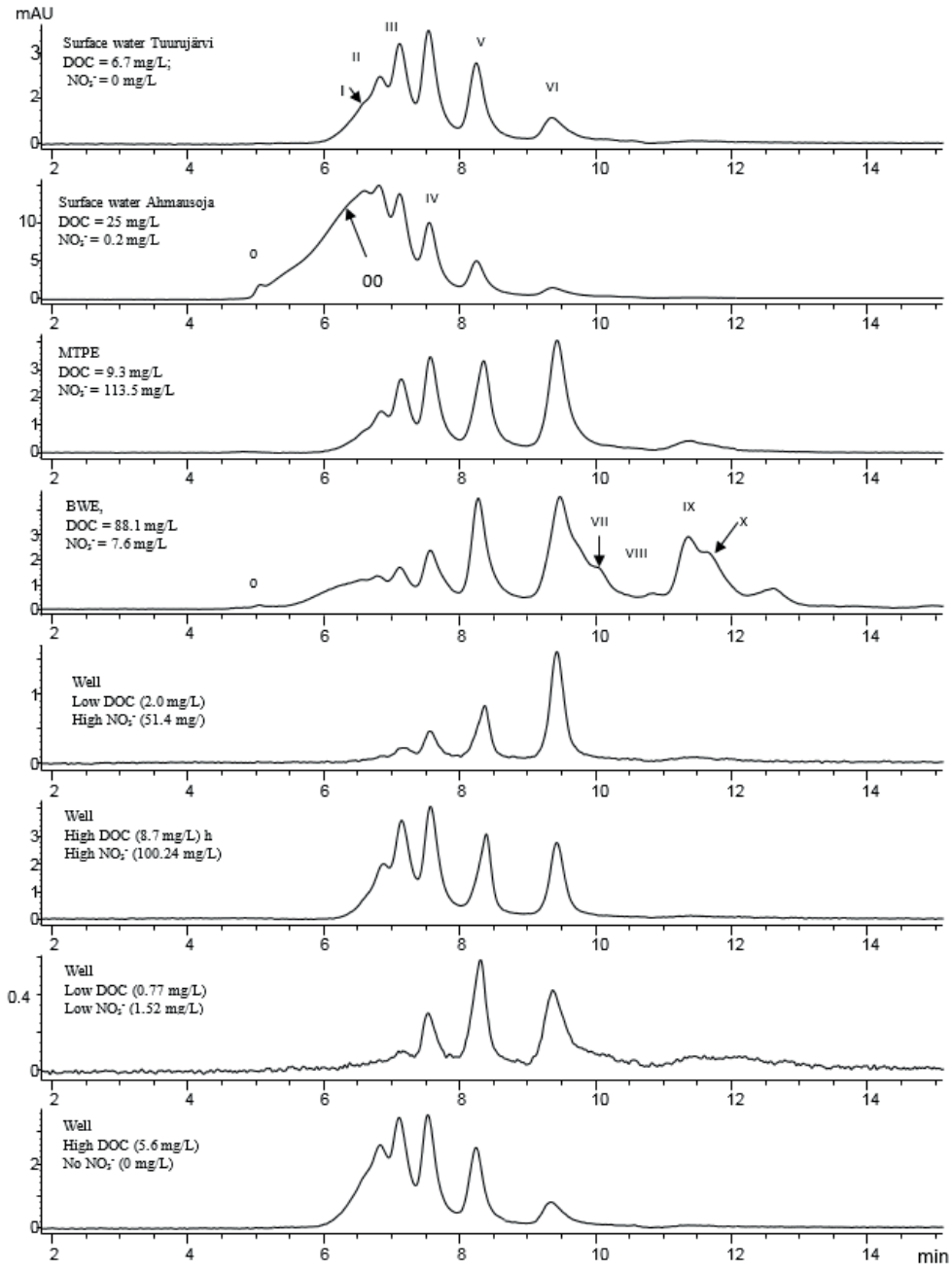


Figure 10. HPLC-SEC-UV254 chromatograms of surface waters, wastewater effluents, and typical well waters; eluent 0.01 M Na-Acetate, injection volume 40 µL

The chromatograms of the water samples revealed that HMW fractions predominate in drains (70-80 % of the total SPH-254), especially in the Jylhäoja mire and forest area. In general, lake waters showed a lower relative amount of HMW (50-70% of SPH-254). Lower DOC values in summer lake samples were associated with decreased HMW and increased IMW fractions, supporting previous studies that have shown degradation of allochthonous compounds into smaller molecules in the epilimnion layer during summer stratification (Osburn et al., 2001; Andersen & Gjessing, 2002). During summer, lakes are stratified and, according to Osborn et al. (2001), intense bacterial activity in the hypolimnion produces HMW organic matter. These macromolecules appear in the upper layers, where samples were taken after autumn mixing, explaining why the winter and spring lake water samples had fractions "0" and "00" and increased HMW. Drinking water chromatograms show that most HMW fractions are eliminated in the treatment plant (Fig. 9). High DOC in drinking water in summer was due to high IMW and the presence of some HMW fractions.

Well waters showed fractions I→VI in the UV-254 chromatograms (Fig. 10). The most interesting finding in this study was that the LMW fraction eluted at around 9.4 min correlated strongly with the nitrate content of the wells (Spearman correlation coefficient 0.729) because of the overlapping of nitrate that still absorbs at 254 nm with simultaneous elution of other organic compounds. None of the fractions VIII→XI in BWE were present in the wells, showing that these fractions do not reach well waters, because they are likely to biodegrade. However, these fractions can be seen in protein-type FLU detection with N-containing compounds transforming into nitrate, which in turn can be detected in well water samples. The maximum, minimum, median, and average SPH values for shallow and deep wells do not differ markedly (Table 10a and 10b). High well DOC and SPH values produced more surface water-like chromatograms with abundant HMW fractions (Fig. 11), whereas in low DOM wells, the dominant fractions were IMW. The anthropogenic influence on wells is seen in the LMW fraction, which shows increased nitrate concentration.

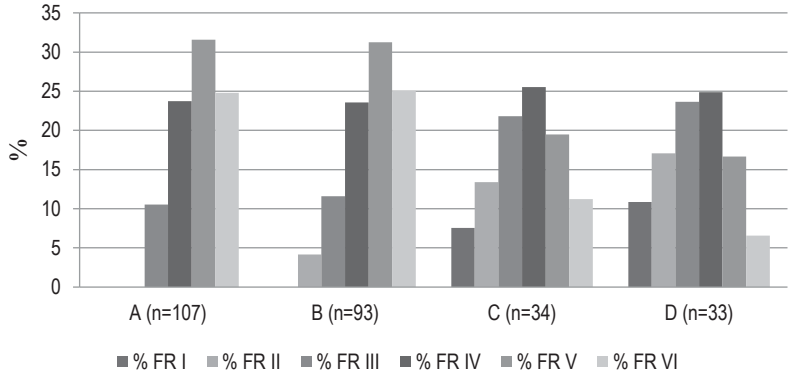


Figure 11. Median percentage composition of the well chromatograms as grouped after DOC values as A: 0 – 1.5 mg/L, B: 1.51 – 3 mg/L, C: 3.1 – 5 mg/L, D: > 5 mg/L; n - number of wells

BWE and GWE produced complex chromatograms (Fig. 12) with a large number of organic molecules coming from wastewater or from microbial cells releasing extracellular polymers. In this study, chromatograms were divided into three main regions. Region 1 peaks are eluted near the void volume, since they are likely to be influenced by ionic repulsion (Ricker & Sandoval, 1996; Pelekani et al., 1999; Bauvier & Koza, 2014). Region 2 peaks are the ones regularly observed in natural and well waters. In this study, region 3 peaks were seen only in onsite WWE. These peaks eluted late, because of secondary interactions (electrostatic- or hydrophobic attractions) between the stationary phase and region 3 molecules. UV-254 detection was best for region 2 peaks of WWE that appeared in all samples. The following sections will further elaborate on wastewater chromatograms.

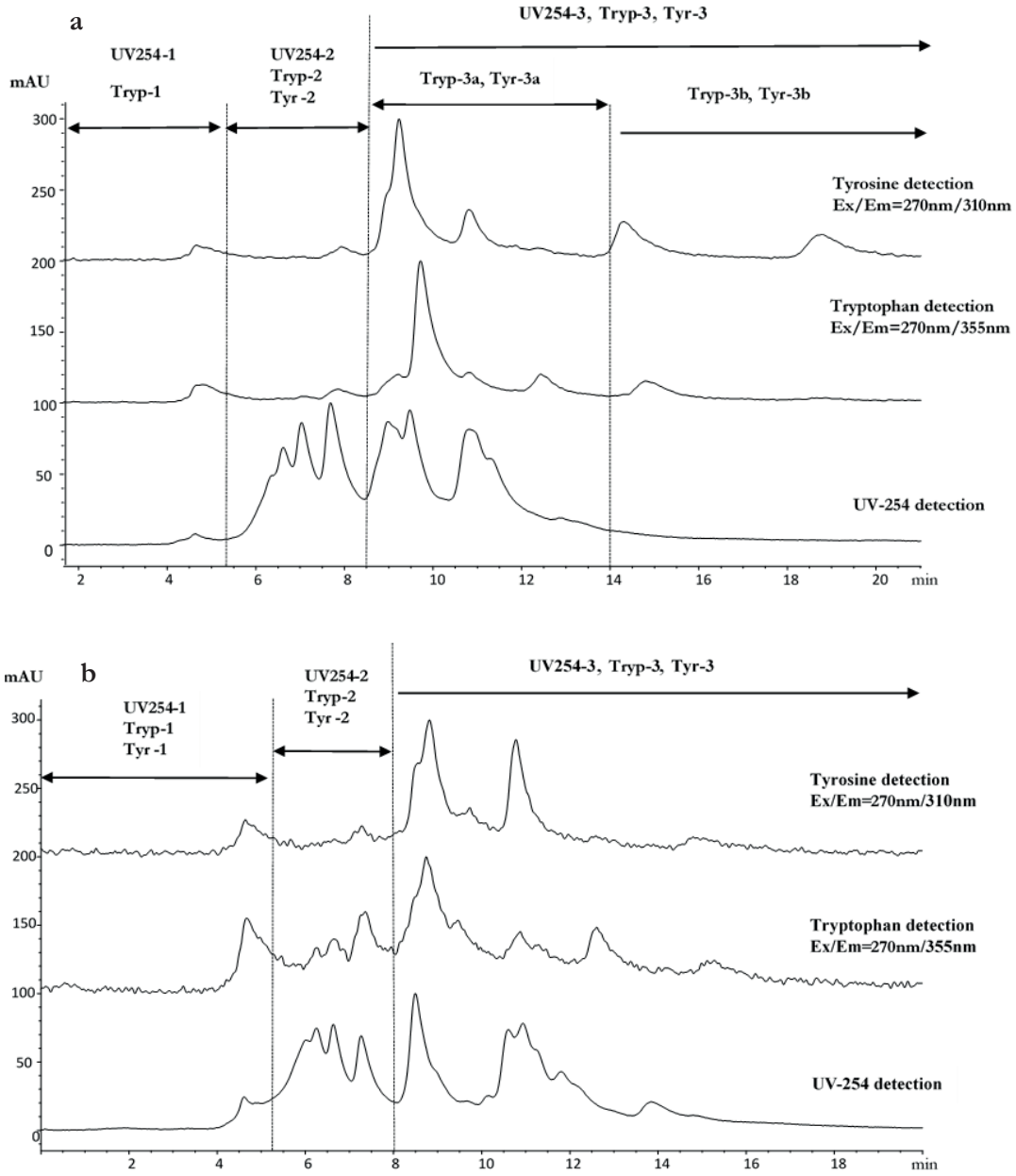


Figure 12. Chromatograms at different detections of blackwater effluent BWE (a) and greywater effluent GWE (b). Eluent: 0.02 M CH_3COONa, pH: 7.2, injection volume 20 µL, *mAU* (milliAmpere Units). Effluent characteristics: BWE: Total-N=164 mg/L, BOD-7=543 mg/L, COD=1500 mg/L ,DOC=170 mg/L; GWE Total-N=35.4 mg/L, BOD-7=509 mg/L, COD=980 mg/L, DOC=244 mg/L)

7.3.3 FLU 270/355 nm (tryptophan-like) and 270/310 nm (tyrosine-like) detection

Well water samples contained low tryptophan-like fractions and even lower tyrosine-like fractions; consequently, protein-type detection was useful neither in characterizing the organic matter in the wells nor in tracking the surface water/wastewater influence on the wells (Szabo & Tuhkanen, 2010).

In the catchment study, the surface water FLU signals were weak, but the SPH of tryptophan-like detection proved capable of indicating the effect of agricultural activities on the drains. It correlated strongly with organic-N (Pearson coefficient 0.759) and also with DOC (Pearson coefficient 0.757) so that the low DOC-content Jylhäoja drain showed consistently higher SPH-tryptophan than the high-DOC content Ahmausoja drain. This is accounted for by the agricultural activities around Jylhäoja, which, according to previous findings, can increase organic nitrogen content in rivers (Rantakaari et al., 2004; Mattson et al., 2005) and tryptophan-like FLU (Baker, 2002).

Protein-type detection proved the most useful in characterizing WWE. Because of the complexity of BWE chromatograms, their region 3 was further divided into region 3-a and 3-b. The subregion 3-b was seen only by FLU detection of BWE. Statistical values of 13 individual ChI (UV254-1, UV254-2, UV254-3, Tryptophan-1 (Tryp-1), Tryp-2, Tryp-3, Tryp-3a, Tryp-3b, Tyrosine-1 (Tyr-1), Tyr-2, Tyr-3, Tyr-3a, Tyr-3b) and their sum values (SPA-UV254, SPA-Tryp, and SPA-Tyr) are given in Table 13.

As expected, GWE samples showed lower ChI values than BWE samples, the means for GWE ranging between 8.4% (Tyr-1) and 62.9% (Tryp-2) of those of BWE. The difference derives from the additional faeces, urine, and toilet paper content in BWE. All BWE and most GWE contained well distinguishable tyrosine- and tryptophan-like fractions in all regions 1, 2, and 3 of the chromatograms. In region 2 (humic), tyrosine signals were weaker than tryptophan signals, and some GWE had no tyrosine-like peaks in this region. Region 3 fractions, eluted after the permeation volume, correlated strongly with conventional indicators, being thus the best candidates for surrogates (Fig. 12, Table 15).

Table 13. Chromatographic indicators (ChI) of blackwater effluents BWEs (a) and greywater effluents GWEs (b)

Chromatographic indicators ChI SPA (mAU*min)	a (BWE)					b (GWE)				
	Sample size	Minimum	Maximum	Mean	Std. Deviation	Sample size	Minimum	Maximum	Mean	Std. Deviation
SPA-UV-254	54.0	23.8	2008.9	725.4	380.2	15.0	101.6	993.3	281.5	214.0
SPA-Tryp	54.0	8.8	1053.3	362.3	251.0	15.0	2.0	263.3	89.6	75.3
SPA-Tyr	54.0	0.0	1066.9	350.5	272.3	15.0	1.0	249.1	60.5	63.1
UV254-1	54.0	0.0	47.2	14.3	10.4	15.0	0.0	36.0	3.5	9.1
UV254-2	54.0	15.5	1075.9	319.1	179.1	15.0	27.0	379.7	130.3	83.3
UV254-3	54.0	8.3	995.1	391.8	233.5	15.0	17.0	577.6	147.6	136.6
Tryp-1	54.0	0.0	117.0	29.0	22.6	15.0	0.0	35.4	5.7	9.4
Tryp-2	54.0	0.0	114.9	37.9	21.3	15.0	0.0	168.0	23.8	42.0
Tryp-3	54.0	8.8	905.6	294.9	218.2	15.0	0.0	173.2	60.0	48.8
Tryp-3a	54.0	8.8	815.3	276.8	202.0	15.0	0.0	0.0	0.0	0.0
Tryp-3b	54.0	0.0	90.3	18.1	23.1	15.0	0.0	0.0	0.0	0.0
Tyr-1	54.0	0.0	70.8	19.8	14.3	15.0	0.0	9.6	1.7	3.1
Tyr-2	54.0	0.0	116.8	20.4	17.3	15.0	0.0	11.3	2.9	3.4
Tyr-3	54.0	0.0	1038.7	315.2	253.4	15.0	0.0	203.2	52.9	54.2
Tyr-3a	54.0	0.0	852.3	226.9	185.8	15.0	0.0	0.0	0.0	0.0
Tyr-3b	53.0	0.0	330.2	85.5	93.6	15.0	0.0	0.0	0.0	0.0

7.4 Chromatographic surrogates for conventional indicators

The correlation between most ChIs and conventional water quality indicators was significant (Table 14 and Table 15). For well waters and surface waters, SPH-254 can be used as a surrogate for DOC and COD, for surface waters SPH-254 can surrogate

DOC, COD-Mn, and TON. The best surrogate for the DOC of WWE are in general tyrosine-like ChI, Tyr-3a for BWE, and Tyr-3 for GWE, which correlate best with DOC. Similarly, BOD-7 correlated best with Tyr-3 for both BWE and GWE, indicating the biodegradability of these fractions. Our result are corroborated by Yu et al. (2014), who reported that tyrosine- and tryptophan-type components in the excitation-emission matrix of municipal wastewater at different purification phases correlated best with DOC. The slightly weaker correlation of BOD-7 is probably owing to variation in microbial diversity from sample to sample (Juanneau et al., 2014).

Table 14. Spearman's rank correlation coefficients between chromatographic indicators and conventional organic water quality indicators, correlations significant at 0.01 level (2-tailed)

	DOC	COD	BOD	Tot-N	TON
Well water, SPH-254	0.777	0.701	-	-	-
Surface water, SPH-254	0.981*	0.916*	-	-	0.8810*
All wastewater effluents, SPA-UV254	0.750	0.738	0.624	0.707	-
All wastewater effluents, SPA-Tryp	0.780	0.671	0.691	0.732	-
All wastewater effluents, SPA-Tyr	0.811	0.670	0.736	0.617	-

*Correlation is significant at the 0.05 level (2-tailed)

UV254-3 is the best surrogate for COD, Tryp-3 for BWE, and SPA-Tyr for GWE. The stronger correlation between SPA-UV254, Tryp, Tyr, and region 3 ChIs of GWE compared to that between BWE and COD can be accounted for by the presence of ammonia from urine in BWE consuming the oxidant in COD determination. Total-N (Kjeldahl) correlated significantly with almost all ChIs, but its best surrogates appear to be tryptophan-like ChIs, in particular SPA-Tryp. The reason for a better correlation between total-N and tryptophan-like ChIs over tyrosine-like ChIs is that a tyrosine molecule has one N atom whereas tryptophan has two. The uncertainties related to Kjeldahl measurement, which include possible N losses during digestion and distillation, might also contribute to the low correlation coefficient values. Interestingly, TDP correlated weakly but significantly with all ChI in BWE but not with any ChI in GWE, probably because of the low amount of TDP left in GWE (Table 15). TDP has the strongest correlation with Tyr-3b, which represents the last two fractions eluted after 14 min for BWE samples

(Fig. 12a), suggesting that these fractions contain most of the dissolved phosphorous in WWE. Moreover, a previous study found that hydrophobic fractions contain most dissolved phosphorous (Qin et al., 2015), suggesting that the interaction between column material and these late eluted peaks may be hydrophobic attraction.

Table 15. Spearman's rank correlation coefficients between chromatographic and other indicators for blackwater effluents (a) and greywater effluents (b). The highest values are in bold type.

Chromatographic indicators ChI, SPA (mAU*min)	a (BWE)					b (GWE)				
	TDP mg/L	Tot-N mg/L	BOD-7 mg/L	COD mg/L	DOC mg/L	TDP mg/L	Tot-N mg/L	BOD-7 mg/L	COD mg/L	DOC mg/L
SPA-UV254	0.338*	0.641**	0.558**	0.680**	0.750**	0.252	0.459	0.873**	0.873**	0.863**
SPA-Tryp	0.496**	**0.665****	0.689**	0.632**	0.818**	-0.195	**0.622***	0.788**	0.818**	0.817**
SPA-Tyr	0.437**	0.518**	0.761**	0.631**	0.870**	0.259	0.356	0.959**	**0.973****	0.945**
UV-254-1	0.274*	0.15	0.438**	0.425**	0.598**	-0.177	0.575*	0.378	0.411	0.469
UV254-2	0.298*	0.556**	0.331*	0.525**	0.482**	0.131	0.300	0.196	0.227	0.157
UV254-3	0.332*	0.627**	0.668**	**0.697****	0.851**	0.145	0.461	0.937**	0.909**	0.949**
Tryp-1	0.391**	0.361**	0.558**	0.572**	0.666**	0.062	0.563*	0.486	0.560	0.498
Tryp-2	0.332*	0.636**	0.517**	**0.697****	0.672**	0.205	0.066	0.632*	0.400	0.446
Tryp-3	0.508**	0.657**	0.708**	0.640**	0.826**	-0.111	0.622*	0.840**	0.847**	0.895**
Tryp-3a	0.485**	**0.661****	0.705**	0.639**	0.832**	no	no	no	no	no
Tryp-3b	0.446**	0.342*	0.539**	0.502**	0.577**	no	no	no	no	no
Tyr-1	0.411**	0.388**	0.521**	0.552**	0.637**	0.073	0.307	0.215	0.350	0.225
Tyr-2	0.280*	0.465**	0.296*	0.338*	0.432**	0.102	-0.537	-0.153	0.064	-0.129
Tyr-3	0.424**	0.479**	**0.791****	0.624**	0.887**	0.235	0.465	**0.968****	0.929**	**0.951****
Tyr-3a	0.308*	0.460**	0.768**	0.636**	**0.893****	no	no	no	no	no
Tyr-3b	**0.563****	0.320*	0.612**	0.497**	0.628**	no	no	no	no	no

** Correlation is significant at the 0.01 level (2-tailed); *Correlation is significant at the 0.05 level (2-tailed)

The above strong correlation coefficients allowed us to assess by linear regression quantitative relationships between the conventional quality indicators and ChI as the best correlates. The results are shown in Table 16. The equations can be used to directly assess conventional indicator values from chromatographic data.

Table 16. Linear regression for quantitative assessment of conventional indicators from chromatographic indicators

Sample name	Equation	HPLC-SEC Conditions	R^2	Number of samples	Variables
Well water	DOC = 0.3323*UV254 + 0.8415	Na-Acetate 0.01M pH =7 injection volume= 40µL	0.7655	267	DOC, mgC/L UV254-SPH, mAU
	COD = 0.3002*UV254 + 0.5441		0.6951	266	COD, mgO_2/L UV254-SPH, mAU
	NO_3^- =0.1884*LMW224		0.9988	9	NO_3^- , mg/L LMW224-PH, mAU
Surface waters (lake + drain)	DOC = 0.282*UV254 + 2.3378		0.9359	30	DOC, mgC/L UV-254-SPH, mAU
	COD = 0.4595*UV254 + 3.5605		0.7887	20	COD, mgO_2/L UV254-SPH, mAU
	TON = 0.008*UV254 + 0.3143		0.7762	15	TON, mgN/L UV254-SPH, mAU
WWE (BWE + GWE)	DOC = 0.1802*Tyr + 33.228	Na-Acetate 0.02 M pH =7 injection volume= 20µL	0.6434	69	DOC, mgC/L Tyr-SPA, mAU*min
	COD = 0.7449*UV254 + 78.177		0.7305	50	COD, mgO_2/L UV254-SPA, mAU*min
	BOD-7 = 0.4562*Tyr + 108.52		0.6167	67	BOD-7, mgO_2/L Tyr-SPA, mAU*min
	TN = 0.1706*Tryp + 24.579		0.5971	63	TN, mgN/L Tryp-SPA, mAU*min
BWE	DOC = 0.263*Tyr-3a + 32.094		0.7553	54	DOC, mgC/L Tyr-3a-SPA, mAU*min
	COD = 1.2278*UV254-3 + 113.98		0.6871	52	COD, mgO_2/L UV254-3-SPA, mAU*min
	BOD-7 = 0.4868 * Tyr-3 + 109.76		0.6767	53	BOD-7, mgO_2/L Tyr-3, mAU*min
	TN = 0.1377*Tryp + 43.888		0.5027	50	TN, mgN/L Tryp-SPA, mAU*min
GWE	DOC = 1.1293*Tyr-3 + 1.0841		0.9417	15	DOC, mgC/L Tyr-3-SPA, mAU*min
	COD = 3.7409*Tyr + 136.32		0.8756	11	COD, mgO_2/L Tyr-SPA, mAU*min
	BOD-7 = 2.7098*Tyr-3 + 21.788		0.8689	15	BOD-7, mgO_2/L Tyr-3-SPA, mAU*min
	TN = 0.0795*Tryp + 2.9136		0.5215	13	TN, mgN/L Tryp-SPA, mAU*min

8 CONCLUSIONS

HPLC-SEC with multiple UV (224 and 254 nm) and FLU (tyrosine- and tryptophan-like) detection has proved a useful analytical tool for detailed characterization of the organic matter content of well waters, surface waters, and simple and complex WWEs. Furthermore, this method allows precise measurement of the nitrate content of well water samples, shows the anthropogenic influence on surface and well waters, and offers quick and reliable assessment of the degree of purification of WWEs. Various chromatographic data can be used to determine the values of conventional water quality indicators, such as DOC and COD of all types of water samples, TON of surface waters, and BOD-7 and TN of wastewater effluents.

The TSK-GEL G3000SW column, Na-Acetate 0.01 M at pH=7 eluent, and an injection volume of 30 μL used in this study produced good separation of DOM up to 8 fractions in surface and well water samples and further up to XI fractions in complex onsite black water effluents (Papers I and II). Secondary interactions cannot be ignored between column gel and sample molecules, especially in high DOC-content surface water and wastewater effluent samples. Therefore, a separate study was necessary to discover the best analytical conditions for analysis of complex WWE. This study (Paper III) revealed that global resolution decreases with increased eluent concentration, and that neutral or basic pH values guarantee better separation than acidic eluents. For high DOC-containing samples low injection volumes resulted in better resolution. UV absorbance and protein-type florescence intensities, in terms of PAs, varied as well with changing eluent conditions. UV254 absorbance was high at neutral and basic eluents, and tyrosine-like FLU intensity decreased significantly as eluent concentration increased. Therefore, an injection volume of 20 μL, up to 0.03 M, and a neutral Na-Acetate eluent proved suitable for WWE analysis (Paper III). Consequently, in our study on onsite WWEs (Paper IV), we used an Na-Acetate 0.02 M at pH =7 eluent and an injection volume of 20 μL.

Secondary interactions hinder the determination of the MWp of fractions, but the well separated peaks and groups of peaks, eluted late due to these interactions, provide quick and reliable nitrate measurement of well waters (Paper II) and

assessment of COD, DOC and, most importantly, BOD-7 of WWEs within half an hour (Paper IV).

The DOM concentration decreases in the catchment from the drains toward the collector lakes, with 35-75% of DOC being eliminated in the terminal lake, which serves as raw water for the drinking water plant. HPLC-SEC-UV254 revealed the nature of DOM in terms of molecular weight fractions, showing that drains contain up to 80% HMW of organic matter and lake waters only 50-60% HMW, its fractions being eliminated to a greater extent within the catchment than the other fractions. Seasonal trends were observed in that drains had high DOC in summer and lakes high DOC in winter and spring. Seasonal increase in DOC was due to increased HMW fractions in the waters. The water treatment plant eliminated HMW fractions from raw water up to 100%, IMW fractions up to 87%, and LMW fractions up to 66%. Seasonal increase in raw water DOM was seen in drinking water as increased IMW and appearance of HMW fractions in the samples (Paper II).

The study on well water (Paper II) UV254 detection revealed that, on average, shallow and deep well water quality differ little in the sparsely populated agricultural areas studied. High DOC-containing well water samples had clear and often dominant HMW fractions, whereas low DOC-containing samples had hardly any HMW fractions but dominant IMW fractions. An LMW fraction, correlating with nitrate, indicates anthropogenic influence.

Nitrate can be precisely calculated from the PH of an LMW fraction detected with UV224, by calibrating the column with nitrate solutions of known concentrations. UV224 detection can also be used to distinguish between aerobically and anaerobically purified WWEs, because nitrate, formed during aerobic treatment of WW, gives a very intense signal at 224 nm at around 9.4 min (Na-Acetate 0.01M eluent, TSK-GEL G3000SW column).

Among the two protein-type detections, tryptophan-type signals, because clearly measurable, can be used to assess surface water. In contrast to UV254 and DOC, tryptophan-like FLU, in terms of SPH, was consistently higher in the drains impacted by agriculture than in those in the mire area, indicating the effect of anthropogenic activities on the drain. Tyrosine-like signals were low in surface waters (Paper I). Because well waters showed very weak protein-type signals, such detection provides no extra information on well water DOM (Paper II)

Paper IV gives good insight into the general quality of septic tank effluents. BWE and GWE samples were clearly different in that BWEs showed high mean values for all conventional indicators, GWE means were 24% of TDP, 24% of Total-N, 66% of BOD-7, 59% of COD, and 66% of DOC-BWE. Chromatograms on WWEs revealed "normal " peaks for surface and well waters though showed also many extra peaks, which were eluted over the permeation volume. By dividing the three chromatograms (UV254, Trypto, Tyro) into 3 regions, we managed to find the best possible surrogates for conventional indicators. Region 3 in the tyrosine and tryptophan chromatograms, representing late peaks eluted over the permeation volume, correlated best with BOD-7, showing that these fractions are biodegradable, do not reach the wells, and thus cannot be seen in well waters. In general, we can confirm that the tyrosine-like chromatograms are the best to assess DOC and BOD-7, tryptophan-like chromatograms are the best to assess TN and UV254, and tyrosine-like chromatograms are the best to assess the COD of WWEs. This study provides regression equations corresponding to the best correlations between ChI and conventional indicators. The equations help reliably calculate DOC, COD, and BOD-7 and to assess roughly the TN of WWEs.

This study highlights the fact that secondary interactions, unwanted in SEC, because they interfere with MSD calculations, can be exploited in nitrate measurement of well waters and BOD assessment of high strength wastewater effluents.

9 RECOMMENDATIONS FOR FURTHER RESEACH

The potential of HPLC-SEC-UV-VIS to monitor wastewater should be further investigated. Further research should focus on systematic monitoring in small and large WWTPs of the DOM and nitrogen content of raw wastewater and purified water after each treatment step. The method would help better understand the functioning of the treatment units in terms of organic matter and nitrogen removal. The method's applicability for analysis of different type industrial wastewaters (e.g., food, pulp, and paper) should also be tested. The focus should be on the method's potential to assess BOD and TN, since the current techniques are laborious, time consuming, or poor in sensitivity and poorly reproducible.

Coupling the analytical setup with online EEMS that could scan the individual peaks or the three groups of peaks (Paper IV) would help better understand the composition and nature of the peaks and in turn help find better surrogates for BOD or TN.

Other types of columns and other eluents should also be tested. The silica gel column used in this study provided good separation of NOM (humic matter). However, in wastewater, the greatest part of DOM consists of polymers originating from microbes or from organic matter anthropogenically released into wastewater. These compounds may separate better in a polymer-based column. Peaks eluted over the permeation volume are likely to be retained within the column by hydrophobic attraction, in which case an organic eluent might elute them earlier. Gradient elution could also be tested during a sample run by gradually replacing an aqueous eluent with an organic eluent. That may ensure elution of more water soluble and less water soluble molecules in a reasonably short time.

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PUBLICATIONS

PUBLICATION I

Natural organic matter from catchment to drinking water: a case study of Pori waterworks, Finland

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Natural organic matter from catchment to drinking water: a case study of Pori waterworks, Finland

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ABSTRACT

In this study Natural organic matter (NOM) characteristics and variations of catchment samples (brooks and collector lakes) from Western Finland, and drinking water produced from the same catchment were examined. Seasonal and spatial NOM variations were followed by means of DOC and HPLC-SEC with UV and fluorescence detection. NOM decreased from drains to lakes by 35 to 75% and from drains to drinking water by 73 to 94%. Drains had a higher NOM content in summer and a lower NOM content in winter and spring. Lakes showed inverse patterns and had a higher NOM content in winter and spring and a lower NOM content in summer. HPLC-SEC separated 8 molecular weight fractions. In drains the HMW fractions represented up to 80% of the NOM, in lake waters HMW fractions accounted for 50 to 70% of the NOM. In drinking water IMW fractions dominated. Increased NOM in raw water during winter was associated with increased IMW fractions and the appearance of HMW fractions in drinking water, DOC increasing from 1.4 mg C/L in summer to 5.8 mg C/L in winter. SPH-Tryptophan correlated with the dissolved organic nitrogen and DOC of the samples. The drain affected by agriculture generally presented higher SPH-Tryptophan values than the unaffected drain.

Key words | catchment, drinking water, fluorescence, HPLC-SEC, NOM

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INTRODUCTION

The concentrations of organic matter in the brooks and lakes of water catchment areas in Finland are generally high and show relatively low seasonal variations, although most of the organic matter in boreal catchments is transported during snow melt in spring and rain events in autumn (Laudon *et al.* 2004; Mattson *et al.* 2005). Organic matter in surface waters in Finland originates mainly from natural sources. Natural Organic Matter (NOM) is generated through microbial processes within the water body or in the surrounding area from plant or algal material. Compositionally NOM is a complex mixture of refractory and biodegradable compounds such as, high molecular weight (HMW) humic and fulvic substances and smaller molecules of proteins, amino-acids, carbohydrates (Leenheer & Croué 2003).

In recent decades, a significant increase in the NOM content of surface waters and a change in NOM properties has been observed in Nordic and Central Europe. This has been associated with changes in catchment area management and changes in anthropogenic impacts on waters (Worral & Burt 2005). The removal of NOM is a priority in a water treatment plant, the efficiency of NOM removal depends on its characteristics and composition (Sharp *et al.* 2006), and these vary spatially and temporally (Hongve 1999; Scott *et al.* 2001; Andersen & Gjessing 2002).Therefore understanding these variations within a particular catchment that serves as a raw water source for drinking water production is crucial for proper water management.

There are several methods for aquatic NOM analysis, High Performance Liquid Size Exclusion Chromatography (HPLC-SEC) coupled with in-line UV detection is a powerful system that gives quantitative and qualitative information on the Molecular Weight Distribution of

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NOM. In the SEC column the components of NOM are separated based on their size. Higher molecular weight compounds penetrate to a lesser extent the pores of the column and are eluted earlier than smaller molecules (Vartiainen *et al.* 1987; Pelekani *et al.* 1999). Fluorescence spectroscopy has been found to be a useful tool in the non-destructive organic matter analysis of water samples. NOM has two distinct classes of fluorophores; humid-like and protein-like (respectively, tyrosine-like and tryptophan-like) fluorophores (Leenheer & Croué 2003). Wastewaters' organic matter shows intensive protein-like fluorescence, tryptophan being the most fluorescent amino-acid in the sewage samples (Baker 2002). Protein-like fluorescence measurements have been successfully applied for the detection of wastewater impact on river waters (Baker 2001) and HPLC-SEC with additional fluorescence detection has been proven to be a useful system for the identification of protein-like components of NOM from groundwater, surface water and wastewater effluent samples as a function of molecular weight (Her *et al.* 2003).

The objective of this study was: to determine NOM characteristics and follow NOM variations within a catchment, in drinking water produced from that catchment, and seasonally by the means of conventional indicators and HPLC-SEC with UV and fluorescence detection. For the study a typical catchment from South Western Finland was selected; the catchment of the lakes Tuurujärvi and Joutsijärvi. Tuurujärvi is a small lake, which is connected to a bigger lake called Joutsijärvi. For the past four decades the quality of Tuurujärvi has deteriorated considerably due to changes in catchment management (forest cutting) and has shown a significant increase in brown colouration, which suggests an increase of NOM.

MATERIALS AND METHODS

Site description

The catchment area of Joutsijärvi and Tuurujärvi in South Western Finland is 117 m^2, which consists of 3.4% agricultural field, 56% forest and 30% mire (Figure 1). Tuurujärvi serves as raw water source for the Pori Waterworks, which supplies the municipality of Pori with drinking water for around 75.000 inhabitants. The majority of the area's agricultural activities (croplands, animal farms) are concentrated around Jylhäoja. In the Ahmausoja sub-catchment forests and drained mire-areas are mainly found. The forests consist of coniferous trees mixed with deciduous stands. The drainage area of Jylhäoja and Ahmausoja makes up 84% of the total catchment. The surface of Joutsijärvi-lake is 840 hectares and Tuurujärvi-lake is 139 hectares. In both of the lakes the brown colouration has significantly increased over the last decades.

The water treatment process at Pori Waterworks consists of coagulation with polyaluminium-chloride, flotation, sand filtration followed by artificial groundwater recharge through basin infiltration with 2 to 3 weeks residence time. Then the drinking water is treated with sodium-hypochlorite and lime.

Sampling and analyses

Our period of study was 2.5 years. Samples were collected from two drains, Jylhäoja and Ahmausoja, from the lakes Joutsijärvi and Tuurujärvi and from the drinking water distribution network. Drain waters were sampled at about 20 cm depth from the surface. Lake water samples were taken from the upper layer of the lake from a depth of 50 to 100 cm. Six sets of samples were collected from December 2006 to May 2008.

The DOC of the samples was determined with a TOC-analyzer SHIMADZU TOC-5000 after the filtration of the samples through a 0.45 μm filter (Whatman). For the HPLC-SEC measurements the samples were first filtered through a 0.45 μm filter (Whatman) and then fractionated with a Hewlett-Packard HPLC 1100 system with a TSKgel G3000SW 7.5 mm × 30 cm column and a diode array UV detector and a fluorescence detector. The UV detection wavelength was set to 254 nm. Sodium acetate of 0.01 M was used as an eluent at a flow-rate of 1 ml/min, the injection volume was 30 μl. This method has been widely used for the characterization of NOM in Finland (Vuorio *et al.* 1998; Nissinen *et al.* 2001; Matilainen *et al.* 2002). In addition, the fluorescence detector was set to detect tryptophan-like fluorescence at excitation/emission wavelengths of 270 nm/355 nm, which is based on previous

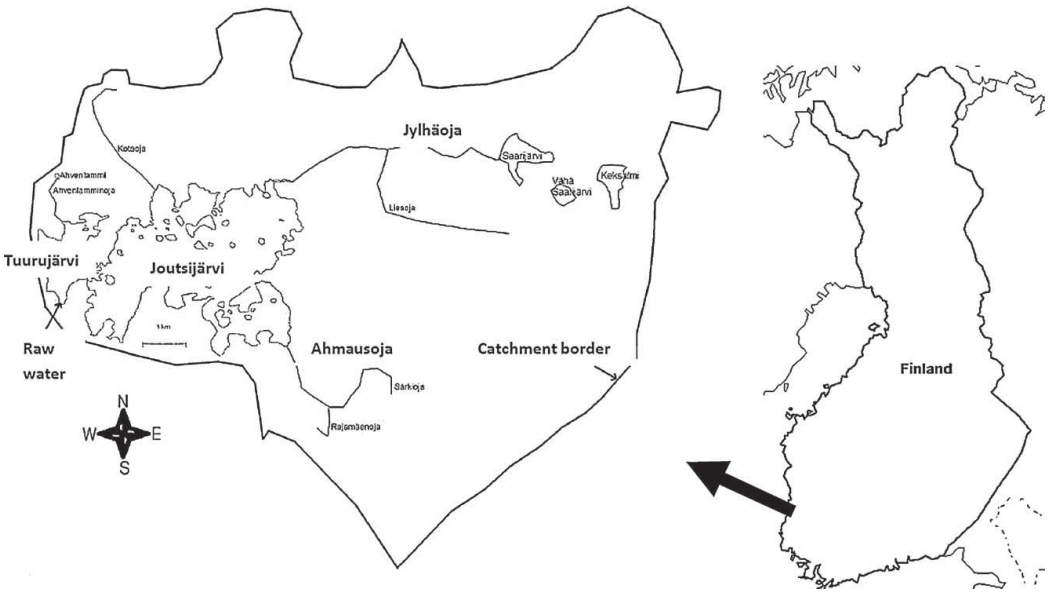

Figure 1 | The catchment area of Joutsijärvi/Tuurujärvi in South Western Finland.

studies (Baker 2002; Her *et al.* 2003), in order to detect the possible effects of wastewater discharge in the drains.

In addition to the DOC and HPLC-SEC data we used the COD-Mn of the catchment samples determined for the years 2000 to 2007, respectively the Total-N and NO23-N values of the studied samples. The Total Organic-N of the samples was calculated as the difference between Total-N and NO23-N.

RESULTS AND DISCUSSION

DOC results

The seasonal variation plot of the KMnO4-number measured over a 6 year period from 2001 to 2006 is presented in Figure 2. This plot shows that the lowest organic content in the drains occurs during winter frost but then increases during the spring snow melt and summer period and reaches a peak in late autumn.

The average, median, maximum and minimum DOC values of water samples are presented in Table 1. The median DOC values of the lake waters examined in this study were 13.2 mg/L for Joutsijärvi-lake and 8.8 mg/L for Tuurujärvi-lake (raw water), which are commonly found in lakes in Finland (Kortelainen *et al.* 1989; Nissinen *et al.* 2001). The median DOC values for the Jylhäoja-drain (19.5 mg/L) and the Ahmausoja-drain (21.3 mg/l) are also quite similar to those found previously in brooks in Finland (20 mg/l–Mattson *et al.* 2003).

However, when comparing the DOC values of the two drains studied, Ahmausoja proved to have a consistently higher DOC than Jylhäoja. According to previous studies, this may be due to the fact that Ahmausoja has more extended mire and forest areas, which contribute greatly to the increase of the DOC export in the boreal catchments (Laudon *et al.* 2004; Mattson *et al.* 2005). DOC concentration decreased from the drains to the lakes, as shown by the fact that 18 to 59% of DOC was eliminated by drainage into Joutsijärvi and 35 to 75% of the DOC was eliminated by drainage into Tuurujärvi (raw water). This is in agreement with the results of previous studies showing that lakes in general act as sinks for DOC upon sedimentation and microbial degradation (Rantakaari *et al.* 2004; Mattson *et al.* 2005). From drains to the drinking water DOC concentration

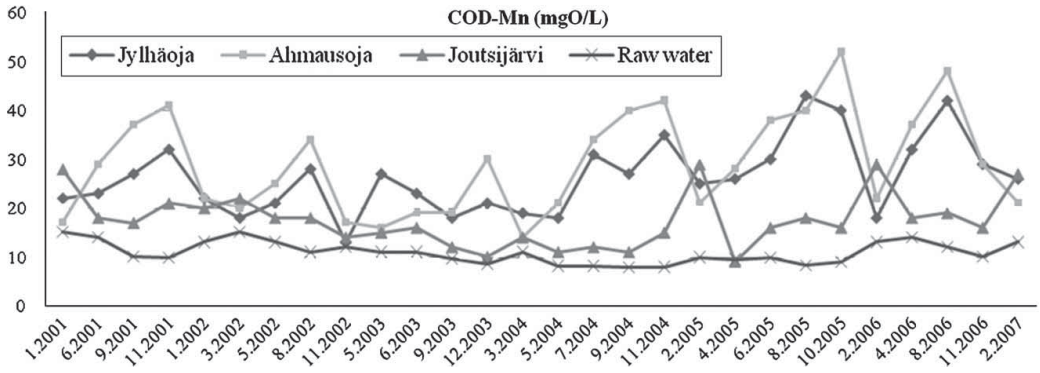

Figure 2 | Chemical Oxygen Demand (COD-Mn) variation in the catchment over a 6 years period (2001–2006).

in both the catchment and water treatment plant was reduced by 73 to 94%. (Figure 3a and b).

With respect to seasonal variation, the DOC of the water samples from the catchment varied significantly across the period of investigation and correspondingly varied in the drinking water. In drains the highest DOC concentrations were found in the August 2007 samples (25 mg/L in Ahmausoja and 20.4 mg/L in Jylhäoja) and the lowest DOC values were measured in April 2007 (15.6 in Ahmausoja and 12.6 in Jyhäoja). These results are in agreement with some previous results which show that soil microbial activity in summer and early autumn is intensified and wet episodes during summer and autumn rainfalls leach freshly produced organic matter from coniferous and deciduous litter (Hongve 1999; Scott *et al.* 2001).

The lakes show a lag in the DOC and KMnO4-number with respect to drains as they generally have their lowest organic content in late summer and autumn. At that time concentrations increase and reach a maximum in late winter (February) (Figures 2 and 3). Similar trends for boreal lake water organic content were observed elsewhere (Matilainen *et al.* 2002; Andersen & Gjessing 2002). Lakes are large water bodies with a high residence time compared to rivers and drains which attenuates organic inputs through various mechanisms such as dilution, sedimentation and transformation (Rantakaari *et al.* 2004; Mattson *et al.* 2005). Andersen & Gjessing (2002) found that transformation through biodegradation in the hypolimnion and photodegradation in the upper layer epilimnion are the main removal mechanism for NOM in a boreal lake and that these processes are at their most intense in summer and early autumn and therefore the lowest DOC content of the lakes generally occurs during this period. Accordingly, decreased bio- and photodegradation during dark and cold winter periods in boreal climates, which follows the autumn mixing of the lake, might be the cause of the increased DOC concentration in the lakes during winter.

The DOC in the drinking water was one magnitude lower than in the drain waters. The seasonal variation of drinking water DOC follows the raw water DOC variation (Figure 3), with the higher organic content of raw water causing a higher DOC in the drinking water. From drinking water samples the highest DOC values were seen in the winter and spring samples from 2008 (5.8 mg/L and 4.8 mg/L), while the

Table 1 | DOC values measured in catchment and drinking water

	Ahmausoja-drain	Jylhäoja-drain	Joutsijärvi-lake	Raw water	Drinking water
Median	21.32	19.5	13.2	8.78	3.12
Average	20.87	18.61	13.5	9.55	3.43
Max	24.97	20.93	17.36	13.9	5.8
Min	15.61	12.64	10.2	6.24	1.42

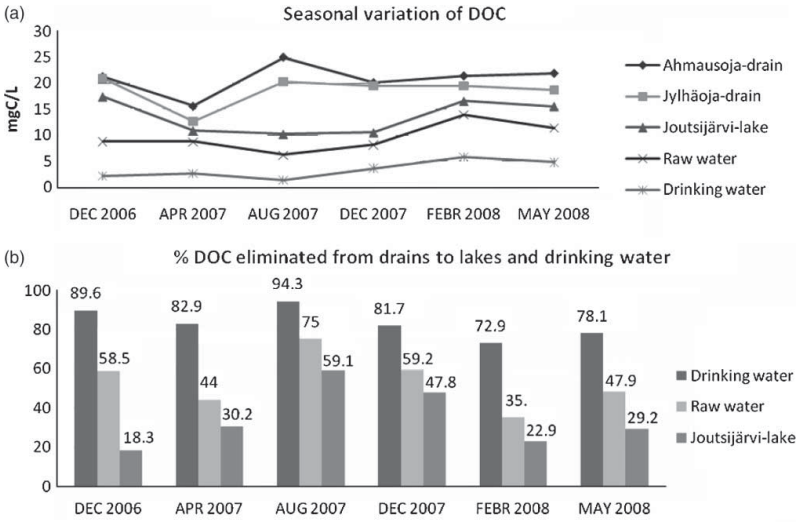

Figure 3 | Seasonal variation and elimination of DOC from drains to drinking water.

lowest DOC was measured in summer 2007 (1.4 mg/L). The summer 2007 measurement also had the highest percentage of DOC elimination at the catchment and water treatment plant (94%). During winter and spring the DOC elimination percentage was consistently lower (72% to 89%) (Figure 3). The results obtained during this research show that the water treatment plant cannot eliminate seasonal variations in DOC from the raw water source. The less effective purification performance during winter and spring can also be explained by the lower performance of some treatment units e.g. flotation and/or filtration during the cold season. Flotation has been found to be sensitive to low temperatures (Matilainen *et al.* 2005) and also biological activity during filtration in winter time has been found to be reduced (Matilainen *et al.* 2006).

HPLC-SEC-UV254 measurements

By HPLC-SEC eight molecular size fractions were separated (Figures 4 and 5). Fraction 0 is an association of smaller molecules and it was present in all of the drain samples and, during winter and spring it was found in lake waters too. Fractions I, II, III and IV are considered to be "High Molecular Weight" HMW fractions; fractions V and VI are the "Intermediate Molecular Weight" IMW fractions and fraction VII is the "Low Molecular Weight" LMW fraction (Vuorio *et al.* 1998; Nissinen *et al.* 2001). For the quantitative characterization of a particular fraction the height of the fraction's peak (Peak height-PH) was used. For the quantitative characterization of the total NOM the sum of the fractions' peak heights (Sum of Peak Heights, SPH-254) was used.

The SPH-254 of the samples correlates with the DOC (Pearson correlation coefficient 0.981) and demonstrates similar patterns to DOC both seasonally and within the catchment.

In drain waters HMW fractions account for 70 to 80% of the total NOM given as SPH-254. In the Ahmausoja-drain water the absolute peak of the HMW and IMW fractions was greater than those of the Jylhäoja-drain, which shows that the larger mire and forest areas around Ahmausoja compared to Jylhäoja contributed to the increased NOM content in Ahmausoja mainly by increasing the amount of HMW and IMW compounds. The lake water samples compared to the drains contain lower relative amounts of HMW (50 to 70%). (Figure 5)

Lake waters have lower HMW and higher IMW peaks in the August 2007 samples and furthermore FR0 and FR I

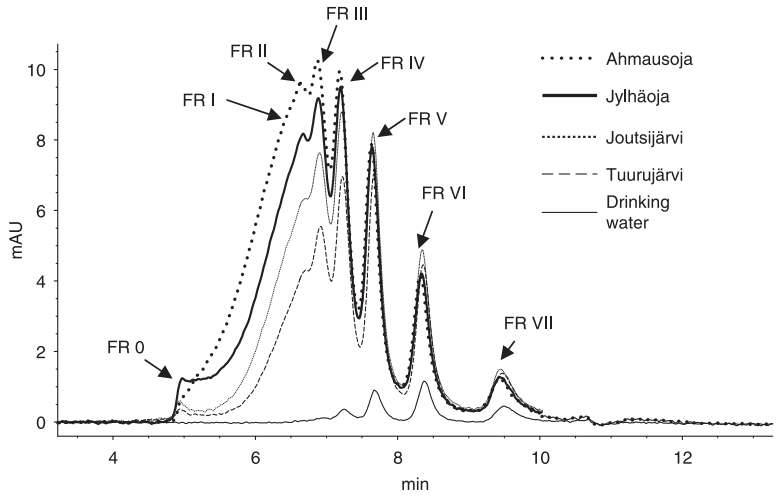

Figure 4 | HPLC-SEC chromatograms from catchment to tap, at UV-254 nm detection.

are absent. It has been shown that during the spring-summer stratification of the lakes in the upper epilimnion layer the HMW, especially allochtonous compounds, are degraded into lower molecular weight compounds through photodegradation (Osburn *et al.* 2001; Andersen & Gjessing 2002). This explains the low amount of HMW and the increased amount of LMW fractions in the summer lake samples of this study. The winter and spring samples have higher HMW peaks and FR 0 and FR I are present in the chromatograms. One explanation for this observation is that during summer and autumn HMW compounds are produced in the hypolimnion through bacterial activity (Osburn *et al.* 2001) and then these HMW compounds enter the upper parts of the lakes during autumn mixing.

In the drinking water the IMW fractions dominate (60 to 70%). The HPLC-SEC-254 measurements obtained from the drinking water samples show that the water treatment chain eliminates most of the HMW fractions, which is in agreement with a series of previous results (Vuorio *et al.* 1998; Nissinen *et al.* 2001; Matilainen *et al.* 2002).

The chromatograms also show that higher NOM amounts in the drinking water in the spring samples compared to the summer samples are due to a higher amount of the IMW fractions V and VI and are due to the presence, although in low amounts, of HMW fractions II and III (Figure 5). These results demonstrate that the water treatment processes (coagulation-flocculation, sedimentation, sand filtration) completely eliminate the HMW fractions 0 and I, which agrees with previous findings (Vuorio *et al.* 1998; Nissinen *et al.* 2001; Matilainen *et al.* 2002, 2005). However, there were residual HMW fractions II, III and IV and relatively high IMW fractions V and VI found in the drinking water when the raw water contained an increased amount of NOM. The marked seasonal changes in the raw waters' NOM composition which were observed in this study by changes in molecular weight distributions (Figure 5), were previously associated with changes in the hydrophobic-hydrophylic character that affected the treatment process (Sharp *et al.* 2006). Since the relative elimination of the fractions from raw water across the research period were roughly the same it can be concluded that seasonal variations in raw water quality can be seen in the drinking water through the variations of the IMW fractions and the appearance of HMW fractions II, III, IV, respectively.

With regard to drains, drinking water samples showed a 95 to 100% elimination of the HMW fractions, an 63 to 90% of elimination of the IMW fractions and a 45 to 76% elimination of the LMW fraction. The April 2007 and February 2008 samples had a lower relative elimination and

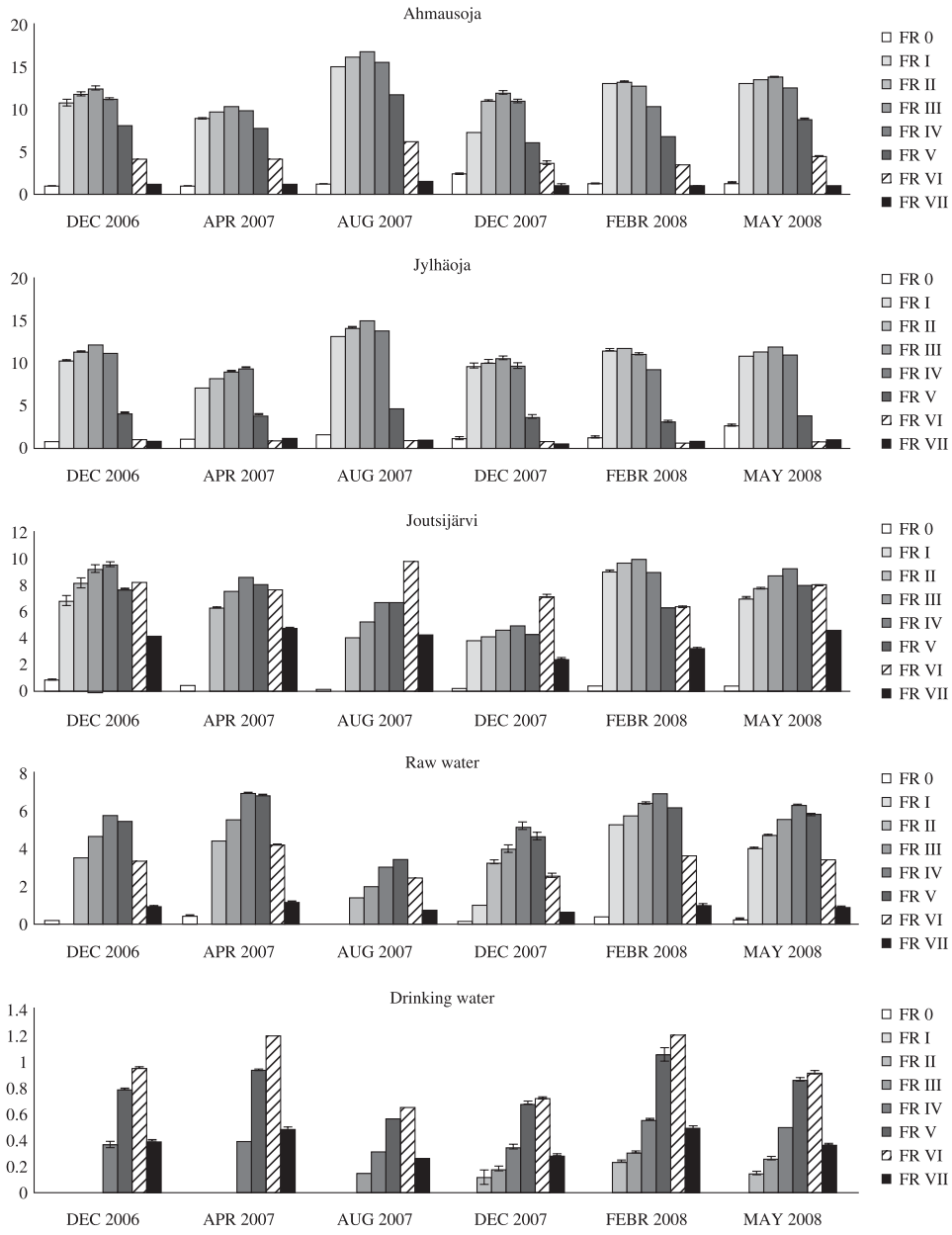

Figure 5 | Seasonal HPLC-SEC Molecular Weight Fractions of water samples from catchment and drinking water.

the August 2007 sample had the highest relative elimination for all of the fractions.

HPLC-SEC-fluorescence measurements

With tryptophan-like fluorescence detection (ex/em = 270/355) only fractions III, IV, V, VI and VII were detected (Figure 6). The fluorescence signals were relative weak and therefore only the sum of the fluorescence-peak heights SPH-Tryptophan was used for further sample characterization.

SPH-Tryptophan correlates rather strongly with the dissolved organic-N content of the samples (Pearson correlation coefficient 0.759) and weakly with NO2,3-N concentrations (Pearson correlation coefficient 0.171). SPH-Tryptophan also correlates strongly with the DOC and SPH-254 (respectively, the Pearson correlation coefficients 0.721 and 0.757). Strong positive correlation between DOC and total organic nitrogen in catchments from Finland has also been found in previous research (Mattson *et al.* 2003). Accordingly, SPH-tryptophan demonstrated similar patterns to DOC and SPH-254 spatially and temporally (Figure 6).

In contrast to the DOC and SPH-254, the Jylhäoja-drain showed 5 to 30% higher tryptophan-like fluorescence than the Ahmausoja-drain, except for the August 2007 and February 2008 values when the fluorescence of Ahmausoja was only slightly higher (Figure 6). Theoretically, this could be explained by the ongoing agricultural activities (croplands and pig farm) in the Jylhäoja-sub-catchment. Previous

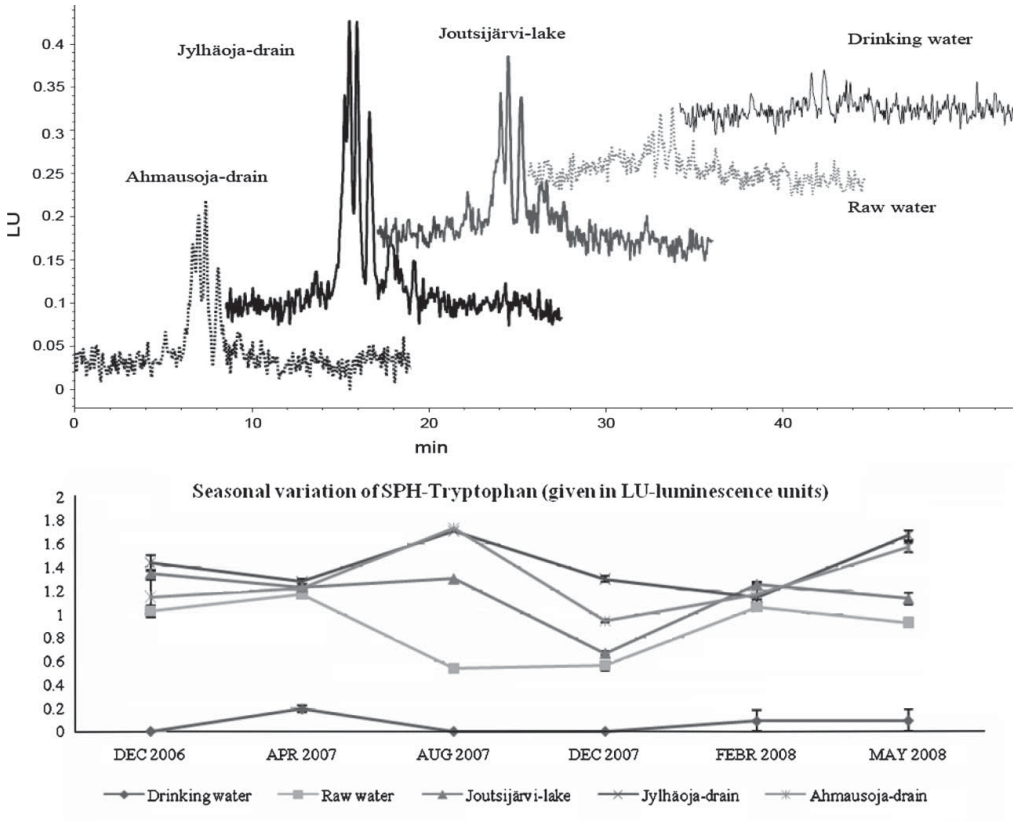

Figure 6 | HPLC-SEC chromatograms from catchment to tap, at FLU-Tryptophan detection and the seasonal variation of SPH-Tryptophan.

studies have shown that organic nitrogen export within a catchment increases with an increase in the proportion of agricultural land (Rantakaari *et al.* 2004; Mattson *et al.* 2005). Additionally, it has also been found that pig slurry has a very intense tryptophan-like fluorescence that should be observable when diluted in rivers (Baker 2002). Hence, it is possible that the increased fluorescence of Jylhäoja compared to that of Ahmausoja reflects the anthropogenic activities around Jylhäoja.

CONCLUSIONS

The amount of NOM was high in the drains and went up to 25 mg C/L then decreased within the catchment to a median value of 9 mg C/L in raw water and reached a minimum value of 1.4 mg C/L in the drinking water produced from it. Correspondingly, 35 to 75% of DOC was eliminated from the drains to the terminal lake (raw water) and 73 to 94% of DOC was eliminated from the drains to the drinking water. The NOM demonstrated seasonal trends whereby the drains had a higher NOM content in summer and a lower NOM content in winter and spring. The two lakes showed inverse patterns and had a higher NOM content in winter and spring and a lower NOM content in summer.

The HPLC-SEC gave detailed information on NOM characteristics in terms of molecular weight composition and separated 8 peaks from the samples. In the drains HMW fractions accounted for up to 80% of the NOM, while in lake waters HMW fractions were about 50 to 60% of the NOM. In drinking water IMW fractions predominated. There was seasonality in the molecular weight distribution of the water samples from the catchment. In the lake waters the IMW fractions were more abundant in summer than in winter and spring, while HMW fractions were higher in winter and spring than in summer. Within the catchment the HMW fractions were eliminated to a greater extent than the other fractions.

The water treatment plant eliminated almost completely the HMW fractions and to a lesser extent the IMW and LMW fractions. There was a seasonal variation in the molecular weight distribution of the drinking water samples; increased NOM in raw water was associated with increased IMW fractions and the appearance of HMW fractions in drinking water. In terms of DOC, this was two to four times higher in the winter and spring samples than in the summer sample. The seasonal increase of NOM in raw water could not be entirely removed by the drinking water treatment system due to the simultaneous effect of low temperatures.

The HPLC-SEC-tryptophan-like fluorescence measurements were interpreted by means of the Sum of the Peak Height-Tryptophan (SPH-Tryptophan). The SPH-Tryptophan followed the same patterns as the DOC and SPH-254. The drain affected by agriculture generally showed higher SPH-Tryptophan values than the non-impacted drain suggesting that traces of wastewater can be detected by HPLC-SEC-fluorescence detection.

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The application of HPLC–SEC for the simultaneous characterization of NOM and nitrate in well waters

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ABSTRACT

The usefulness of HPLC–SEC for the characterization of well water quality and the identification of surface-water or wastewater percolation into wells was studied. In total, 267 private wells from rural areas of Finland, two surface waters and two wastewater effluents were analyzed for organic matter (Dissolved Organic Carbon (DOC)) and nitrate with conventional methods. High Performance Liquid Size Exclusion Chromatography (HPLC–SEC) was also used for NOM (Natural Organic Matter) and nitrate analysis. High DOC values were found occasionally in both shallow and deep wells. HPLC–SEC with UV-254 detection separated 6 fractions in the wells studied: three High Molecular Weight (HMW) fractions, two Intermediate Molecular Weight (IMW) fractions and a Low Molecular Weight (LMW) fraction. The LMW fraction is an overlap of LMW organic compounds and nitrate. In wells with a high DOC content the chromatograms were typically "surface water"-like, with HMW fractions clearly present and often dominant. Anthropogenic influence on wells was seen in the increase of LMW fraction VI, which shows an increased nitrate concentration. Nitrate concentration can be determined with precision by HPLC–SEC using UV detection at 224 nm.

1. Introduction

Private water-wells are an important source of domestic and drinking water for rural populations worldwide, but are often quality impaired due to the infiltration of pollutants in groundwater bodies. Agricultural activities and improperly functioning onsite sanitation systems present a common threat to well water quality. In Finland, after several extensive nationwide surveys in the 1990s it was found that only 40% of the private wells fulfilled all of the hygienic and technical requirements for drinking water. Increased colour was a frequently found problem (Korkka-Niemi, 2001; Lahermo et al., 2001), and there were often high nitrate concentrations especially in inland wells surrounded by intensive agricultural activities (Mitikka et al., 2005). One area of importance these studies did not cover completely was the organic matter content of water samples. The organic matter amount is conventionally shown by quality indicators like colour and the $KMnO_4$ number. However, colour, besides brownish coloured organic matter, also indicates Fe-content and the $KMnO_4$ number, in addition to organics, includes all of the inorganic reducing components. Therefore, surrogate water quality indicators, like dissolved organic carbon (DOC) or natural organic matter (NOM) could provide better information on the amount and nature of the organic matter of water samples.

Natural organic matter (NOM) in waters is derived from plant or terrestrial (allochtonous) and algal (autochthonous) sources and it is composed of higher molecular weight refractory humic and fulvic substances, as well as lower molecular weight proteins, organic acids, carbohydrates and other possible anthropogenic compounds (Leenheer and Croué, 2003). As numerous previous studies have revealed, dissolved NOM composition and structure depends on its source. There are considerable differences in composition and in the spectroscopic and chromatographic characteristics of NOM derived from surface waters, soil seepages, wastewater effluents and ground waters (Frimmel and Abbt-Braun, 1999; Ma et al., 2001; Her et al., 2003). Thus, NOM measured in well water samples could be a useful indicator of surface water or wastewater percolation into a well.

HPLC–SEC (High Performance Liquid Size Exclusion Chromatography) coupled with in-line UV detection is a commonly used for the analysis of NOM. This system provides quantitative and qualitative information on the molecular weight (MW) components of the NOM of the different types of raw water samples without the need for any laborious pre-treatment. In the SEC column the molecules are separated according to their size, the lower molecular size compounds better penetrate the pores of the gel and are eluted later than the higher molecular size compounds (Pelekani et al., 1999; Zhou et al., 2000). However, during size exclusion secondary electrostatic and hydrophobic interactions can occur between the column gel and solute molecules, which can interfere with the determination (Wu et al., 2003).

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The objectives of this study were to characterize the NOM of the wells, surface waters and wastewater effluents by HPLC–SEC; and to assess the applicability of HPLC–SEC chromatograms in identifying those wells affected by surface water, wastewater or the leaching of organic soil matter.

2. Materials and methods

2.1. Wells, surface water and wastewater effluent samples

In total 267 wells were analysed. Of those 193 were classified as shallow wells (either dug, or a spring with a depth of 0.8–10 m) and 74 were classified as deep wells (borehole with a depth of 20–133 m). The well water samples were taken from four regions within Finland: from the Pirkanmaa area, from West Finland, from East Finland and from Southern Finland. The samples were taken between 2003 and 2007 and during all seasons. The analysed wells are private wells from places of permanent residence, or summer cottages, located in sparsely populated areas. Most of the sites are affected by intensive present or past agricultural activities. Each well was sampled once. The samples were collected in one litre polyethene bottles and kept in a refrigerator until the analysis. The analyses were carried out within 5 d of collection.

Two surface waters and two wastewater effluents were also analysed in order to determine and compare the surface water type and the wastewater type chromatograms. The surface water samples were taken from a lake (Tuurujärvi), and a drain (Ahmausoja). Both are in a catchment area in Western Finland, near to the municipality of Pori. The drain collects water from a relatively pristine forested swamp area and has high humus content brown water. Tuurujärvi is a lake that collects water from both pristine and also agricultural areas and is the raw water source for the municipality of Pori. One of the wastewater effluents was a secondary effluent from the municipal wastewater treatment plant of Tampere municipality, which has more than 200 000 inhabitants. The second wastewater effluent sample was taken from an onsite septic system where three septic tanks treat the black water of a small household. The sample was taken from the third septic tank that is the endpoint of the purification, the effluent then being discharged into a collector ditch.

2.2. Methods

2.2.1. Organic content

The dissolved organic carbon (DOC) of the samples was determined with a TOC-analyzer SHIMADZU TOC-5000. For the HPLC–SEC measurements the samples were first filtered through a 0.45 μm filter (Whatman) and then fractionated with a Hewlett–Packard HPLC 1100 system, a TSK-GEL G3000SW 7.5 mm × 30 cm column and diode array UV detector. The detection wavelength was set to 224 nm and 254 nm. Sodium acetate of 0.01 M was used as eluent at a flow-rate of 1 mL min^{-1} and the injection volume was 30 μL. This method was used in a series of previous studies on NOM (Peuravuori and Pihlaja, 1997; Matilainen et al., 2002). The HPLC–SEC column was calibrated with narrow standards of polystyrene sulfonates (PSS), Na-salts of molecular weight 210, 4300, 6800, 13 000 Da (Fluka) and 2200, 3600 Da (Polymer Standard Services). The void volume and permeation volume was determined with Blue Dextrane (1 000 000 Da) and acetone (58 Da). In order to improve the HPLSEC accuracy, the results of the PSS standards were combined with those of acetone, as recommended by Zhou et al. (2000).

From 2003 to 2007 three TSK-GEL G3000SW columns were used. Each column was separately calibrated with narrow PSS standards and acetone.

2.2.2. Nitrate concentration

The nitrate concentration was measured using the liquid chromatography of ions. The instrument used was a Dionex D-120 ion-chromatograph with an AS9-HC separating column and a conductivity detector. The eluent used was a buffer solution with a concentration of 12 mM Na_2CO_3 and 5 mM $NaHCO_3$ with a flow-rate of one millilitre per minute. The nitrate concentration of 67 wells was also determined by HPLC–SEC, as described below.

2.2.3. Statistical analyses

Statistical analysis was performed to determine the relationships among the measured water quality indicators. SigmaStat for Windows Version 3.00 (SPSS Inc.) was used. The goodness of fit to the normal distribution of the data was tested with the Kolmogorov–Smirnov test. Since none of the examined parameters showed normal distribution, Spearman's rank correlation coefficient was used to evaluate the strength of association between the parameters. A $P < 0.01$ was considered statistically significant.

3. Results and discussion

3.1. General quality of the wells

The general characteristics of the shallow and deep bedrock wells are given in Tables 1a and b.

With regard to the health related quality defects, the same range of shallow and deep wells had nitrate concentration over the guideline value of 50 mg L^{-1}. Moreover, levels of nitrate over 10 mg L^{-1} are considered already elevated (Gelberg et al., 1999), accordingly in this study 39% of the shallow wells and 33.8% of the deep wells had increased nitrate concentrations. The natural level of nitrate in Finnish groundwaters is less than 0.2 mg L^{-1} (Korkka-Niemi, 2001), therefore it is likely that the increased nitrate levels in groundwater are entirely due to anthropogenic activities. Although less organic carbon was expected in deep wells, the results show almost the same range of DOC in both types of wells.

3.2. HPLC–SEC analysis of the samples

3.2.1. Calibration of HPLC–SEC column

The calibration equations obtained by linear regression are not presented here. The resolution of this SEC system had previously been found to be good enough to use only the peak heights of the separated fractions (Peuravuori and Pihlaja, 1997), thus in constructing the calibration curves we used the retention times that corresponded to the peak maximum of the eluted standard. Using the calibration equations the molecular weights corresponding to the peak maximum of the resolved NOM fraction (MWp) were determined.

3.2.2. Nitrate analysis by HPLC–SEC

UV 224 nm measurements revealed a very intensive peak at about 9.2 min corresponding to the LMW fraction in the samples with increased nitrate concentration. Since nitrate absorbs intensively at 224 nm, pure Nitrate solutions of 6, 10, 20, 30, 50, 100, 150 and 200 mg L^{-1} were run through the SEC system in order to elucidate whether nitrate is eluted at the LMW peak retention time or not. The chromatograms presented in Fig. 1 clearly demonstrate that nitrate is indeed eluted at the LMW peak retention time (9.4 min). The same trend can be observed at UV 254 nm (Fig. 1b) when fraction VI also reveals strong and significant Spearman correlation coefficients with nitrate (Table 2). However, the peak heights measured at 254 nm are more than two orders of magnitude less than those measured at 224 nm.

Table 1a
Descriptive statistics of the quality indicators of shallow wells analysed in this study.

Indicator (recommended or guideline values)*	Size	Mean	Std Dev	Max	Min	Median	10%	90%	Over guideline (*n* (%))
NO_3^- (mg L ^{1})	193	14.22	24.59	219.94	0	4.88	0.12	41.59	
(<50 mg L^{-1})									13 (6.7%)
(>10 mg L^{-1})**									75 (39%)
DOC (mg L^{-1})	193	2.5	2.08	10.90	0	1.84	0.74	5.61	
(<2 mg L^{-1})									87 (45%)
SPH-254 nm (mAU)	193	4.77	5.39	29.9	0.06	2.61	0.84	13.12	

Table 1b
Descriptive statistics of the quality indicators of deep wells analysed in this study.

Indicator (recommended or guideline values)*	Size	Mean	Std Dev	Max	Min	Median	10%	90%	Over guideline (*n* (%))
NO_3 (mg L^{-1})	74	11.83	21.33	126.36	0	0.59	0	39.96	
(<50 mg L^{-1})									5 (6.8%)
(>10 mg L^{-1})**									25 (33.8%)
DOC (mg L^{-1})	74	2.41	2.52	11.78	0.01	1.51	0.5	5.32	
(<2 mg L^{-1})									28 (37.8%)
SPH-254 nm (mAU)	74	5.41	6.77	30.99	0	2.5	0.56	12.08	

NO_3^- - nitrate concentration; *n*-number of wells; DOC - dissolved organic carbon; SPH-254 – sum of peak heights determined by HPLC–SEC at 254 nm detection; mAU – milliAmpere units).
* According to WHO, 2004.
** Increased value set according to Gelberg et al., 1999.

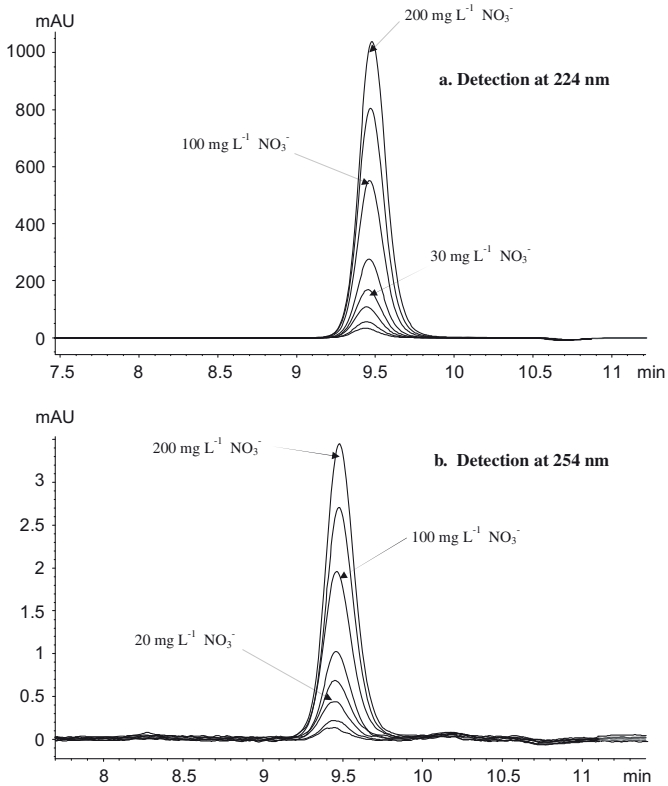

Fig. 1. Chromatograms of pure nitrate solutions, concentrations: 6, 10, 20, 30, 50, 100, 150 and 200 mg L^{-1} at detection wavelengths 224 nm (a) and 254 nm (b).

The earlier than expected elution of nitrate ions in the HPLC–SEC has previously been observed on a Macrosphere GPC 60 column with 50 mM ammonium acetate eluent solution (pH 5.6) in the analysis of heavy metal ions in cucumber plants (Mihucz et al., 2000). This anomalous nitrate ions behaviour can be explained by the structure of the stationary phase in the column

Table 2
Spearman's rank correlation coefficients between the organic water quality indicators and nitrate; bold type: significant correlations, with $P < 0.01$.

	DOC	SPH-254	FR I	FR II	FR III	FR IV	FR V	FR VI
NO_3^-	0.277	0.274	0.072	0.001	0.074	0.174	0.28	0.729
DOC		0.777	0.61	0.683	0.721	0.759	0.787	0.591
SPH-254			0.756	0.861	0.919	0.967	0.953	0.698
FR I				0.86	0.801	0.738	0.642	0.276
FR II					0.917	0.862	0.766	0.391
FR III						0.922	0.842	0.468
FR IV							0.941	0.591
FR V								0.725

DOC-dissolved organic carbon; SPH-254 nm - sum of peak heights determined by HPLC–SEC at 254 nm detection, given in milliAmpere units; FRI → FRVI – peak heights of the fractions separated by HPLC–SEC, at 254 nm detection, in mAU.

and the pH ionic strength of the eluent. The TSK-GEL G3000SW column is filled with porous silica that is chemically bonded with a hydrophilic material. The eluent pH is 7.1 and the ionic strength of the eluent is low. According to Varga et al. (2000) at a high enough pH and a low ionic strength the hydrophilic silica-based stationary phase is negatively charged. In this case, electrostatic repulsion forces act between the stationary phase and the negative nitrate ions, causing the early elution of the latter.

Although these secondary ionic interactions are unwanted in SEC, in the case of well water analysis the simultaneous information on both organic content and nitrate is beneficial.

In order to determine the nitrate concentrations of well water samples by HPLC–SEC, we calibrated our column for both 224 nm and 254 nm detection using the eight nitrate solutions mentioned earlier (concentration range of 0–200 mg L^{-1} NO_3^-), obtaining regression lines of $R^2 = 0.999$ (224 nm) and $R^2 = 0.992$ (254 nm).

To validate the HPLC–SEC method, we compared the nitrate concentrations in 67 well water samples (all run through the SEC column calibrated for nitrate) as obtained by IC (concentration range 0–100 mg L^{-1} NO_3^-) with those obtained by HPLC–SEC-224 nm (Fig. 2a) and by HPLC–SEC-254 nm (Fig. 2b).

The regression equations show that the nitrate concentrations determined by HPLC–SEC are higher than those determined by IC. This is due to the fact that other compounds than nitrate are eluted at the "LMW fraction" elution volume and these compounds also absorb at the detection wavelengths 224 nm and 254 nm, respectively. Thus, one can assume that the "LMW fraction" signal is the result of the overlapping of nitrate and other compounds eluted at the same time.

The regression equations also show that nitrate concentrations determined by HPLC–SEC-224 nm are only slightly higher (>1.2 mg L^{-1}) than those determined by IC. Meanwhile, the nitrate

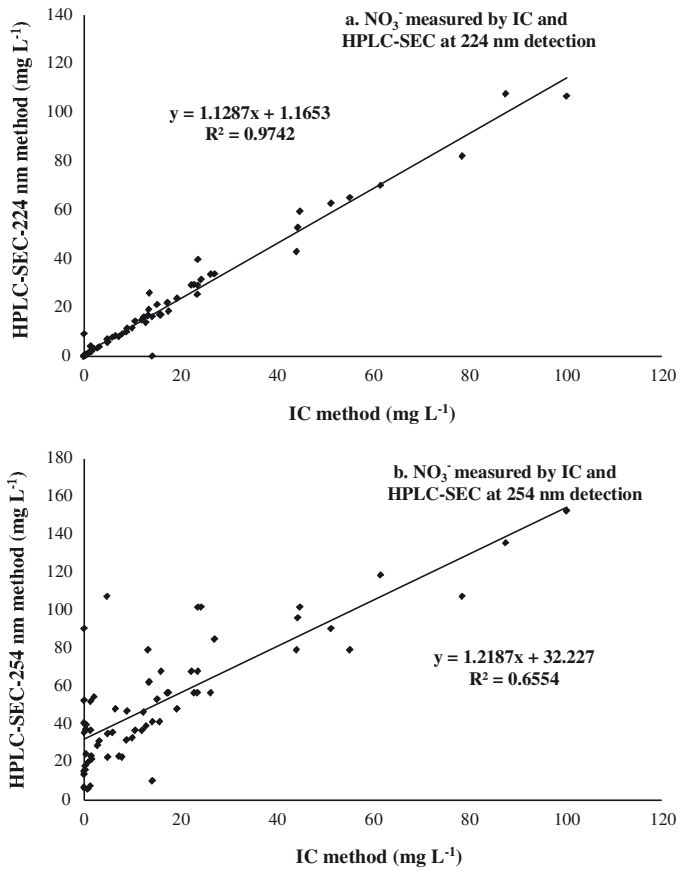

Fig. 2. A comparison of the NO_3^- measurements obtained by the HPLC–SEC and the IC methods ($n = 67$ wells).

concentrations measured by HPLC–SEC-254 nm are significantly higher (>32 mg L^{-1}) than those measured by IC. This is explained by the fact that the absorption of the LMW organics eluted at 9.4 min is nearly the same at the two detection wavelengths but the absorption of nitrate decreases significantly from 224 nm to 254 nm, consequently the interference of LMW organics is stronger at the HPLC–SEC-254 nm determination of nitrate.

Based on these results it can be concluded that the nitrate concentration of water samples can be determined with precision by HPLC–SEC-224 nm.

3.2.3. Organic matter analysis by HPLC–SEC

The chromatograms represent the molecular size distribution (MSD) of NOM in the corresponding sample. In well waters six

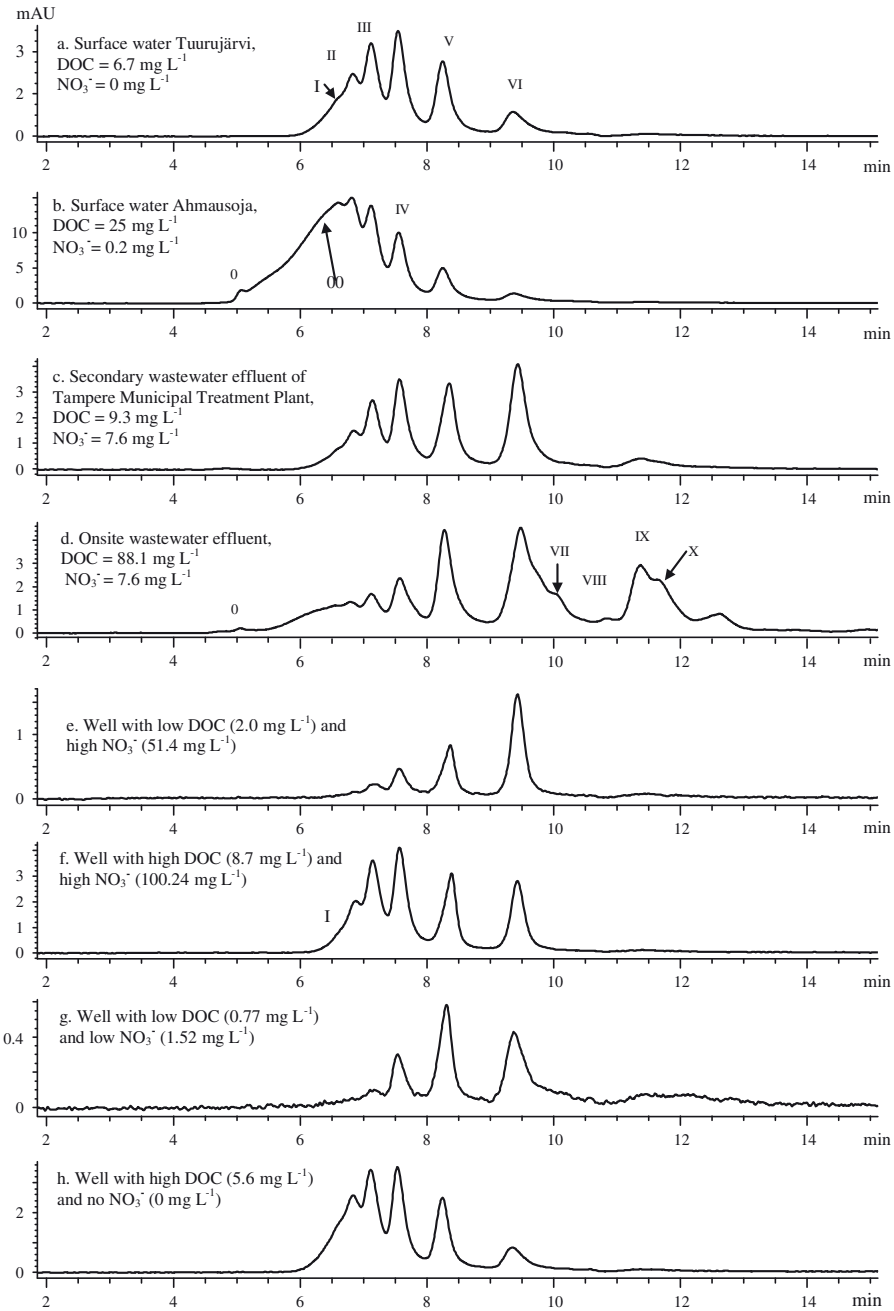

Fig. 3. Chromatograms of surface waters, wastewaters and some typical chromatograms of well water samples.

fractions were separated by HPLC–SEC and noted as FRI → FRVI (Fig. 3). Fractions I (MWp 2600–3300 Da), II (MWp 2200–3000 Da) and III (MWp 1900–2500 Da) are considered high molecular weight fractions (HMW), fractions IV (MWp 1400–1800 Da) and V (MWp 800–1200 Da) are intermediate molecular weight fractions (IMW), and fraction VI (MWp 350–470 Da and nitrate) is the low molecular weight fraction (LMW) (Vuorio et al., 1998; Nissinen et al., 2001; Matilainen et al., 2002). As described above, the LMW fraction VI eluted at 9.4 min is an overlapping of the LMW organic fraction and nitrate. The peak molecular weights MWp (molecular weights corresponding to the peak maximum) determined for fractions I → VI are of the same magnitude as those determined in two other studies with HPLC–SEC and PSS calibration (Christensen et al., 1998; Pelekani et al., 1999). However, a closer comparison of the obtained MWp values cannot be made since, in the other studies mentioned, other types of columns and eluents were used and the NOM were pre-treated by XAD-resin fractionation (Christensen et al., 1998) and ultrafiltration (Pelekani et al., 1999). The pretreatment of NOM might cause change in the initial NOM structure and subsequently change its SEC behaviour (Frimmel and Abbt-Braun, 1999; Pelekani et al., 1999). Several additional peaks were separated from the high humus containing surface water drain Ahmausoja (DOC = 25 mg L^{-1}) (Fig. 3b) and from the wastewater effluents (DOC = 9.3 in municipal effluent and DOC = 88.1 - mg L^{-1} in onsite effluent) (Fig. 3c and d). The first peak eluted (FR 0) was present in both the Ahmausoja and the onsite wastewater effluent samples and was eluted near to the void volume at 5.2 mL (Fig. 3b and d). According to our experience this fraction would seem to be constantly present in high humus content surface waters and in unprocessed onsite wastewater effluents. We presume that this fraction contains an association of smaller molecules present in the organic matter washed from soil into the collector ditches or present when wastewater effluents, such as septic system effluents are not processed properly. Another possibility is that this fraction has a lower molecular weight than that calculated, but is eluted earlier than expected due to repulsive ionic forces (Pelekani et al., 1999; Varga et al., 2000).

There is a striking difference between the chromatograms of the secondary wastewater effluent of MTP (DOC = 9.3 mg L^{-1}) (Fig. 3c) and the onsite wastewater effluent (DOC = 88.1 mg L^{-1}) (Fig. 3d). In the onsite wastewater effluent sample a series of LMW peaks, FR VIII → FRXI were additionally eluted, while only FR IX, in low amounts, was present in the MTP effluent. These fractions are non-humic fractions that can be seen with the aid of protein-type fluorescence detection (Szabo and Tuhkanen, 2007), and other interactions than size exclusion may contribute to their late elution, e.g. hydrophobic adsorption by the gel or ionic attraction between the solute and gel. The high DOC and complicated chromatogram of the onsite wastewater effluent used in this study show that the onsite treatment system is not sufficient for the proper purification of domestic wastewater.

None of the fractions VIII → XI found in onsite wastewater effluent were seen in any of the well water samples, which suggests that these fractions are biodegradable and do not reach ground waters. However, these fractions can be seen later on as an increased concentration of nitrate in the wells as they are protein-type fractions and their decomposition in the soil leads ultimately to nitrate formation.

For the quantitative characterization of the separated fractions and the total NOM the heights of the eluted peaks (PH-peak height) and their sum (SPH-sum of peak heights) were used.

According to chromatograms in the drain water with high humus content the high molecular weight HMW fractions I, II and III predominate (Fig. 3b). The drain's catchment lake Tuurujärvi contains only moderate amounts of NOM (Fig. 2a). Correspondingly, fractions I and II are found in lower proportions and fractions III (HMW) and IV, V (IMW) are the predominant ones in the Tuurujärvi chromatogram. Similar results were obtained in a number of previous studies conducted on lake waters that are used as raw water for drinking water production in Finland (Vuorio et al., 1998; Nissinen et al., 2001; Myllykangas et al., 2002; Matilainen et al., 2002). The chromatogram of municipal wastewater effluent (Fig. 3c) shows LMW fraction VI to be dominant, partly because of the high nitrate concentration of the effluent that overlaps with other low molecular weight organic compounds. These compounds are likely to be low molecular weight hydrophilic acids that were found to dominate in municipal wastewater effluents (Imai et al., 2002).

The maximum, minimum, median and average values of the fractions and SPH for shallow and deep wells are given in Tables 1a and b. These values show no significant differences between the shallow and deep wells. However, there are significant differences between the samples with different NOM amounts (Figs. 3 and 4). Wells with high SPH and high DOC values show surface water type chromatograms where the high molecular weight HMW fractions I, II and III are clearly present (Figs. 3f and h 4 – MaxSPH). Wells with low SPH and low DOC values do not have HMW fractions I and II, and in most of the cases fraction III is present only in very low amounts or absent (Fig. 4 – MinSPH; Fig. 3e

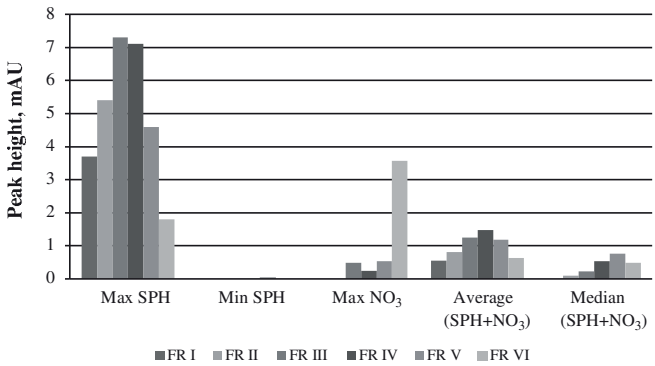

Fig. 4. Molecular Size Distributions (MSD) of representative well water samples. *Max SPH*-well with maximum sum of peak heights. *Min SPH*-well with minimum sum of peak heights *MaxNO*$_3$-well with maximum nitrate concentration. *Average* (*SPH* + *NO*$_3$)-well closest to average sum of peak heights and average nitrate concentration. *Median* (*SPH* + *NO*$_3$) – well closest to median sum of peak heights and median nitrate concentration.

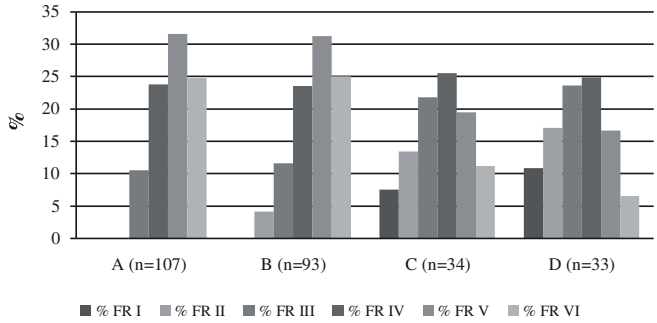

Fig. 5. Median percentage composition of the well chromatograms as grouped after DOC values as (A) 0–1.5 mg L^{-1}, (B) 1.51–3 mg L^{-1}, (C) 3.1–5 mg L^{-1}, (D) >5 mg L^{-1}; *n* – number of wells.

and g). The increase in fraction VI shows an increased nitrate concentration rather than increased LMW organic compounds (Fig. 3e and f) and the chromatograms of those wells resemble the chromatogram of properly processed municipal wastewater effluent.

In order to ascertain the composition of the chromatograms of the wells with low, intermediate and high humic content, the wells were grouped according to their DOC content into four groups (1–1.5 mg L^{-1} DOC, 1.51–3 mg L^{-1} DOC, 3.1–5 mg L^{-1} DOC, >5 mg L^{-1} DOC). The median percentage compositions of the well chromatograms presented in Fig. 5 show that in well waters containing low DOC the dominant fractions are the IMW and LMW fractions IV, V and VI, while the HMW fractions I and II are present in very low amounts or absent. The same results were obtained by Nissinen et al. (2001) for groundwaters and artificially recharged groundwaters. However, with the increase of DOC the relative proportions of HMW fractions I, II and III increase and the dominant fractions gradually become fractions HMW III, and IMW IV, V (group 3.1–5 mg L^{-1} DOC) and fractions HMW II, III and IMW IV for the last group of wells with >5 mg L^{-1} DOC.

As expected, the Spearman rank correlation coefficients between the DOC and SPH-254 and the DOC and the fractions are strong and significant (Table 2). LMW fraction VI shows a weaker correlation with the other organic parameters due to the interference of nitrate, as described above.

In summary, the wells demonstrate a wide variety of HPLC–SEC chromatograms, high DOC content is associated with surface water type chromatograms and these wells are clearly affected by the leaching of humic matter from soil and surface water. Wastewater percolation is not so evident from the HPLC–SEC data, since no LMW fractions, VIII → XI, typically characteristic of unprocessed wastewater effluent were observed in any of the wells. However, an increase in the LMW fraction VI shows an increased nitrate concentration in wells, which reveals an anthropogenic influence.

4. Conclusions

High DOC values can be found in both shallow and deep wells occasionally. HPLC–SEC with UV-254 detection separated six peaks in the wells: HMW fractions FR I, II, III; IMW fractions FR IV, V and the LMW fraction FRVI that represents the low molecular weight organic compounds and nitrate. When compared with the well water chromatograms, two additional HMW fractions were found in the surface water samples and wastewater effluents and four additional LMW fractions were eluted from onsite wastewater effluent. The onsite wastewater effluent used in this study had a complex chromatogram compared to the municipal wastewater effluent, which suggests that an onsite septic system is not sufficient for the proper purification of domestic wastewater. In low DOC containing well waters the chromatograms have low peak heights, the dominant fractions are the IMW and LMW fractions and the HMW fractions are often absent. In wells with higher DOC the chromatograms obtained are typical of "surface water" and have peak heights sometimes higher than those of surface water samples. In such cases HMW fractions are clearly present and often dominant. Those wells are clearly affected by surface water or the leaching of humic matter from the soil. Wastewater percolation is not so evident from the HPLC–SEC data. The increased LMW fraction VI shows an increased nitrate concentration that is due to anthropogenic influence. Nitrate concentrations can be determined with precision by HPLC–SEC using UV detection at 224 nm. Overall, it would appear that the HPLC–SEC is an effective tool for the rough estimation of the leaching of organic matter into wells and the determination of anthropogenic influences on a well.

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PUBLICATION III

HPLC-SEC: a new approach to characterise complex wastewater effluents

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HPLC-SEC: a new approach to characterise complex wastewater effluents

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ABSTRACT

This work investigates the use of HPLC-SEC to characterise dissolved organic matter (DOM) of complex wastewater effluents. A silica-based column, sodium acetate eluent and multiple detections were employed: UV-254 absorbance for humictype, and tryptophan-like (Ex/Em = 270/355) and tyrosine-like (Ex/Em = 270/310) fluorescence for protein type compounds. Effects of eluent pH, eluent ionic strength and injection volume on separation efficiency were tested. Humic-type and protein-type fractions were clearly differentiated and eluted within and out of calibration range. Eluent ionic strength had the greatest influence on global resolution; the lowest eluent concentration of 0.01 M produced the best separation for all wastewater effluents tested at any detection. UV-254 absorbance was higher at neutral and basic eluent pH while tryptophan-like fluorescence depended on the sample composition rather than on the eluent pH or ionic strength. Tyrosine-like fluorescence decreased significantly with the increase of eluent ionic strength. Accurate molecular weight measurements could not be done, the separation being influenced by secondary interactions, but could be approximated using separate calibrations with sodium salts of polystyrene-sulfonates and protein standards. The results show that this method is suitable for determining DOM in wastewater at low eluent concentrations (up to 0.03 M), at neutral or slightly basic pH.

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Fluorescence; UV-254; proteins; humic; ionic strength; DOM; tryptophan; greywater

1. Introduction

Dissolved organic matter (DOM) and N-compounds in natural water systems are key indicators of pollution caused by diffusion of wastewater effluents (WWE) into natural waters. DOM and N-compounds in wastewater are routinely quantified using chemical oxygen demand (COD) and biochemical oxygen demand (BOD) and Kjeldahl methods. However, these detection methods, though reliable in characterising the global organic content, have several drawbacks: they do not provide information on the components of WWE, use harmful chemicals (COD, Kjeldahl) and are time consuming (BOD, Kjeldahl).

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Therefore, different analytical techniques are needed for more efficient quantitative and qualitative characterisation of DOM in WWE and natural waters.

Aqueous, high performance, size exclusion liquid chromatography (HPLC-SEC) is widely applied in analysing mixtures of organic compounds using, for example, silica-based columns, as they provide good separation of proteins and other biomolecules [1,2] and natural organic matter [3–5]. Theoretically, separation in HPLC-SEC is based solely on the hydrodynamic volume of the solute molecule. Therefore, the main application of HPLC-SEC is to determine molecular weight (MW) of organic compounds based on calibrated columns with globular proteins or narrow standards of polystyrene-sulfonates or polyethylene-glycols [1–9]. Many studies have shown that HPLC-SEC with a silicagel column has good separation potential regardless of the nature of the interaction between the stationary phase of the column and the solute molecules [e.g. 1–4]. Besides the above application, SEC can also be used to assess simultaneously natural organic matter and nitrate in well water samples [10].The eluents used in HPLC-SEC are salt solutions of a given ionic strength and pH value (usually a mixture of Na_2HPO_4 and NaH_2PO_4 at pH 6.8) [7,11]. However, better separations can be achieved with CH_3COONa solution [4]. UV/VIS is suitable for detecting organic matter containing aromatic groups ('humic-type' compounds), while fluorescence detectors are generally employed in tandem with UV/VIS [7,11] to measure simultaneously protein-type compounds.

HPLC-SEC has been applied to study DOM in environmental samples, but very few studies exist on its use to analyse complex WWE, mainly in monitoring the performance of municipal wastewater treatment processes, where most of the initial organic matter of the wastewater had already been removed [11,12]. The purpose of this study was to test the applicability of HPLC-SEC in the analysis of differently treated (aerobic and anaerobic) complex WWE. Our main aim was to test the separation potential of a silicagel column using CH_3COONa as an eluent. We also tested the usefulness of multiple UV and fluorescence detectors for simultaneous detection of humic-type and protein-type compounds of WWE. A secondary aim was to determine the analytical conditions (injection volume, eluent conditions) that provide the best separation of organic matter from high-, medium- and low-strength WWE (containing high, intermediate and low amount of dissolved substances).

2. Experimental

2.1 *Instrumentation and procedures*

WWE samples were analysed using a Hewlett-Packard HPLC 1100 equipped with a silica gel-based TSK-GEL G3000SW 7.5 × 30 cm column (TOSOH Bioscience) and two detectors: an UV/VIS HP 1100 Series Diode Array Detector and an HP 1100 Series Fluorescence Detector. The settings for the UV/VIS and fluorescence detectors were detection wavelength at 254 nm (UV-254 nm) and two excitation/emission wavelength set for tryptophan-like detection (Ex/Em 270 nm/355 nm) and tyrosine-like detection (Ex/Em 270/310). The excitation/emission wavelengths were selected based on previous studies [13,14]. The eluent flow rate was 1 ml/min.

The effects of the eluent ionic strength on separation was done using CH_3COONa solutions of 0.01–1.0 M, prepared from $CH_3COONa{\cdot}3H_2O$ (M = 136.08 g/mol, MERCK) in

deionised water (Milli-RO 30 plus (Millipore)). The eluent pH values were adjusted with NaOH (4 M) or HCl (32% and 6.4%). For the eluent ionic strength experiments, the pH of the eluent was kept constant at 7.0. In addition, we used 0.02 M CH_3COONa at various pH values (5.5–8.2) to test the effects of the eluent pH on the chromatographic separation. For each elution condition, the void and permeation volumes were determined with Blue Dextran (MW = 10^6 Da, FLUKA, Switzerland) and acetone (MW = 58 Da), respectively. The samples were filtered through a 0.45 µm filter (Whatman) prior to analysis. The integration of the chromatograms was made with the software 'Agilent ChemStation for LC systems' and the peaks were visually identified. The sum of the area of the peaks was used to measure chromophoric DOM moieties.

The column was calibrated for each elution condition separately using the following standards: PSS (sodium salts of polystyrene-sulfonates, MW 210 Da, 4300 Da, FLUKA, Germany), 6800 Da, 13000 Da (FLUKA, Switzerland), 2200 Da (Polymer Standard Service, Germany), and proteins such as tyrosine (MW = 181 Da), tryptophan (MW = 204 Da, Sigma-Aldrich, Japan), β-Lactoglobulin (from bovine milk, MW = 18000 Da, Sigma, USA) and bovine serum albumin (MW = 66000 Da, Sigma, USA), as well as vitamin B12 (MW = 1400 Da, Sigma-Aldrich, China). For each eluent condition, three calibration equations were determined: with PSS standards only (using acetone to determine the permeation volume); with Protein standards only (using tryptophan to determine permeation volume); and with all standards (PSS + proteins).

2.2 *Chromatographic response function and global resolution*

The quality of the chromatographic separation was assessed using global resolution term from the expression of the chromatographic response function (CRF) [Equation (1); 15].

$$\mathrm{CRF} = \sum_{\mathrm{I}-1}^{N-1} \theta_{\mathrm{s,I}} + N - \left(\frac{t_{\mathrm{L}} - t_0}{t_{\mathrm{L}}}\right), \tag{1}$$

where $\sum_{\mathrm{I}-1}^{N-1} \theta_{\mathrm{s,I}}$ is the global resolution (GR); $\theta_{\mathrm{s,I}}$ is the estimate of the resolution between two consecutive peaks; N is the number of peaks; t_{L} is the retention time of the last peak eluted within the SEC calibration range; t_0 is the retention time of the first peak eluted within the SEC calibration

The term $\theta_{\mathrm{s,I}}$ between two adjacent peaks was calculated with Equation (2).

$$\theta_{\mathrm{s,I}} = 1 - \frac{H_{\mathrm{v}}(t_{\mathrm{I}} - t_{\mathrm{s}})}{(t_{\mathrm{v}} - t_{\mathrm{s}})|H_{\mathrm{I}} - H_{\mathrm{s}}| + H_{\mathrm{s}}(t_{\mathrm{I}} - t_{\mathrm{s}})}, \tag{2}$$

where H_{v} is the height of the valley and t_{v} is the retention time of the valley between the two peaks; H_{s} and t_{s} are the height and retention time of the first peak, respectively; H_{I} and t_{I} are the height and retention time of the second peak, respectively [15].

2.3 *WWE samples*

Five WWE were analysed in this study, four onsite effluents from private households and a secondary effluent from Tampere Municipal Wastewater Treatment Plant (MTP).

Table 1. Characteristics of the wastewater effluents, single measurements.

	pH	Conductivity, µS/cm	Total N, mg/l (Kjeldahl)	Chemical oxygen demand (COD), mg/l ($K_2Cr_2O_7$ titration)	Biological oxygen demand (BOD), mg/l (Oxitop)	Dissolved organic carbon (DOC), mg/l (Shimadzu****)
Mixed 1*	7.96	1893	174	1177	501	269
Mixed 2*	6.82	1396	112	1253	498	149
Grey 1**	7	822	7	333	166	45
Grey 2**	6.66	569	11	272	93	22
MTP***	7.5	-	31[a]	31.3[a]	3.3[a]	9.3

*Onsite (1, 2) effluents of mixed, household washing and toilet wastewater.
**Onsite (1, 2) effluents of grey, household wastewater (excluding toilet water).
***Secondary effluent of Tampere Municipal Wastewater Treatment Plant.
****Result is the average of four consecutive injection.
[a]Data published by Tampere Water in summer 2008.

Table 1 shows the main characteristics of the samples. Grey 2 and Mixed 2 WWE were not tested in the injection volume runs.

3. Results and discussion

The void volume, determined by Blue Dextran, was constant at 5 ± 0.01 ml, while the permeation volume, determined by acetone, was constant at 12 ± 0.1 ml, indicating that these two compounds follow the rules of size exclusion, regardless of variation in the pH or increase in the eluent ionic strength.

Multiple detections revealed different chromatograms (Figure 1(a) and (b)). The effluent from aerobically treated municipal wastewater showed less fractions than other effluents used in this study, all eluted within the calibration range for both detections (Figure 1(c)).

The chromatograms can be divided into three main regions: I, II and III (Figure 1(a)). Region I represents the 'humic-type' fractions found in surface- and well water samples, detected by UV-254 [4,10]. Region II has fractions still within the calibration range that were detected by both UV-254 and fluorescence; tryptophan- and tyrosine-type fractions being clearly differentiated.

The fractions eluted before and around the void volume are also seen by both UV-254 and the protein-type detections, but the protein-type peaks have slightly longer retention times (Rt). Region III covers the fractions eluted after the permeation volume. Based on the SEC theory and previous studies, fractions eluted before the void volume and over the permeation volume do not obey the rules of SEC but are influenced by secondary interactions. In particular, the fractions eluted before the void volume contain compounds subjected to repulsive electrostatic interactions between the molecules and column material; the fractions eluted after the permeation volume are retained by ionic attraction or hydrophobic interaction between the solute molecules and the column material. Therefore, we cannot draw any conclusions about the MW of these fractions [1,2,6–8].

3.1 *Calibration and MW determination*

The constants of the calibration equations, determined by the least square fit at different eluent conditions, are shown in Table 2. With the exception of Blue Dextran and

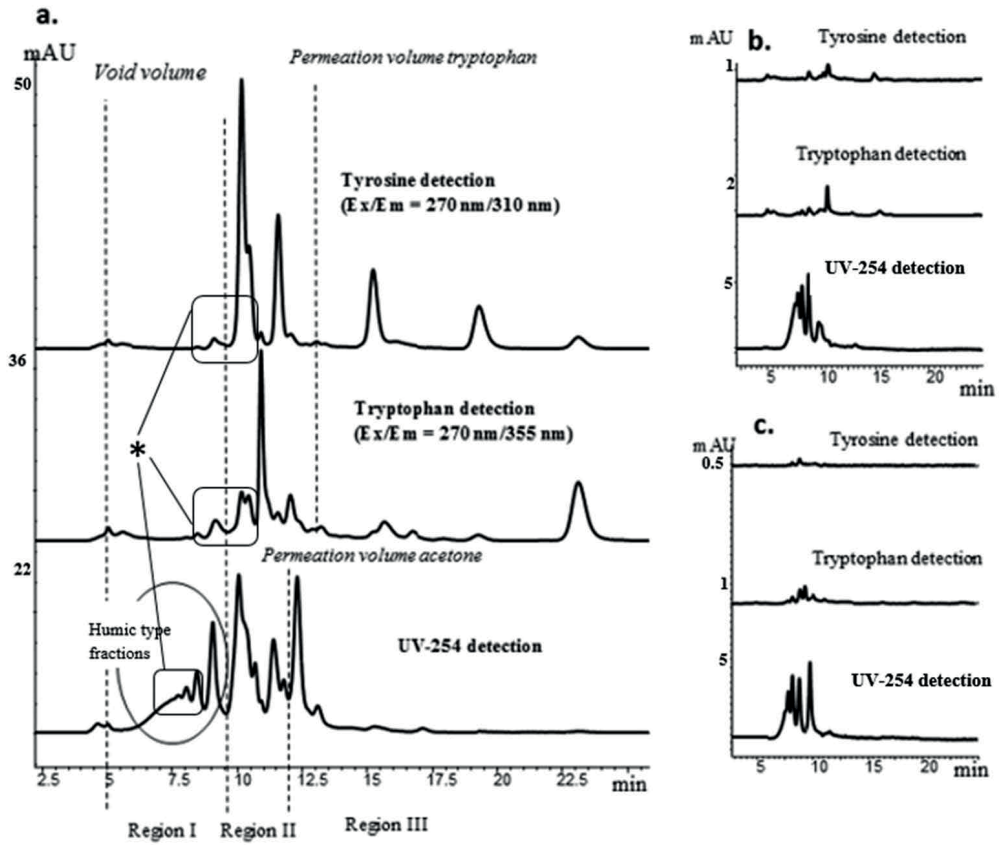

Figure 1. Chromatograms at different detections of mixed wastewater effluent (Mixed 1, a), grey-water effluent (Grey 1, b), municipal wastewater effluent (MTP, c). Eluent: 0.02 M CH_3COONa, pH: 7.2, mAU: milliAmpere Units. *The four fractions used to assess the effects of varying eluent conditions.

acetone, the Rt of all the calibration standards changed more or less with the eluent pH and concentration. With respect to eluent pH, the Rt of the calibration standards decreased with increasing eluent pH up to 6.8, with the longest Rt at pH 5.5. At eluent pH above 6.8, the Rt of the PSS standards remained stable and those of the proteins increased slightly. The shapes of the PSS standards and the peak areas of the rest of the standards also varied with eluent pH. The variation in the shapes of the PSS peaks was observed at acidic eluent pH up to 6.8, implying changes in the structure of these standards. At pH > 7, the calibration standards had higher peak areas suggesting that the deprotonated forms of the standards used absorbed better UV-254 nm and showed higher tryptophan- and tyrosine fluorescence, confirming previous results [16–22].

With respect to eluent concentration (ionic strength), the Rt shifted upwardly with increase in eluent concentration for all the calibration standards. Generally, the PSS standards showed higher shift in Rt (up to 5 min, range 0.01–1 M) than the protein standards. Similar results were reported in literature for both humic- and protein-type solutes, and were attributed to reduced ionic repulsion and increased hydrophobic attraction at the increased eluent ionic strength [1,2,3,6,8,9,11,23,24].

Table 2. SEC column calibration with polystyrene-sulfonates (PSS) and protein standards.

IS (M)	PSS			PSS + Proteins			Proteins		
				log(Mw) = a − b*Rt					
	a	b	R^2	a	b	R^2	a	b	R^2
0.01	5.383	0.3023	0.9537	5.4188	0.2623	0.7609	6.2157	0.3054	0.9009
0.02	5.6927	0.3094	0.9622	5.8285	0.2875	0.7876	6.7705	0.3445	0.9179
0.03	5.9835	0.3261	0.9519	6.1672	0.3124	0.7915	7.1934	0.3777	0.9146
0.05	6.3659	0.3484	0.933	6.6202	0.3466	0.8083	7.6071	0.4106	0.9084
0.1	6.765	0.369	0.8922	7.1477	0.385	0.8174	8.1016	0.4496	0.9013
0.2	7.0845	0.3828	0.8402	7.5777	0.4141	0.8201	8.4097	0.4725	0.8931
0.5	7.2312	0.3796	0.7606	7.8165	0.4243	0.798	8.5382	0.4768	0.8776
1	6.9901	0.3455	0.6797	7.6482	0.3975	0.7573	8.3734	0.4542	0.8578
pH	PSS			PSS + Proteins			Proteins		
				log(Mw) = a − b*Rt					
	a	b	R^2	a	b	R^2	a	b	R^2
5.5	6.2897	0.3265	0.8648	6.8257	0.3564	0.7653	8.4134	0.4782	0.9009
6.2	6.0214	0.3303	0.9622	6.2744	0.3167	0.7869	7.453	0.4035	0.9180
6.8	5.6696	0.3176	0.9616	5.723	0.2777	0.7174	7.0185	0.3688	0.9277
7	5.6927	0.3094	0.9622	5.8285	0.2875	0.7876	6.7705	0.3445	0.9179
7.2	5.6387	0.3038	0.9557	5.7809	0.2802	0.7659	6.859	0.3551	0.9201
7.7	5.6315	0.3035	0.9583	5.7689	0.2803	0.7789	6.7478	0.3471	0.9159
8.2	5.6367	0.3028	0.9539	5.8588	0.2971	0.817	6.6334	0.3447	0.9004

R^2: regression coefficient; a and b: constants in calibration equations; Mw: molecular weight (Da); Rt: retention time (min); IS: eluent ionic strength (mol/l)

As to the variations in the Rt of the calibration standards with eluent pHs and eluent concentrations, the question arises as to what extent these variations are due to size-exclusion (changes in the size of the molecule due to changes in the eluent conditions) or due to secondary interactions. Separate calibrations with PSS standards (excluding eluent IS > 0.2 M) and protein standards gave higher values for R^2 (coefficient of determination); therefore, it is practical to use separate calibrations for protein-type and UV-254 signals. Considering the separate PSS and Protein calibrations, R^2 values over 0.9, at a wide range of eluent concentration and various pH, indicates that the size-exclusion mechanism is preserved. However, an increase in eluent concentration over 0.05 M leads to a decrease in R^2 as hydrophobic interactions between calibration standards of different MW and column material start to act more and more 'unevenly'. pH effect on R^2 (and therefore on SEC mechanism) is less significant, since only pH of 5.5 produces an R^2 value less than 0.9 (Table 2).

3.1.1 *Determination of the MWs of wastewater fractions*

The MWs corresponding to peaks maxima were calculated only for the fractions eluted within the calibration range. To determine the effects of varying eluent condition on the calculated MWs of the fractions, we compared the sum of the first four sample fractions after the void volume (both humic-type fractions at UV-254 detection and protein-type detection, after 7 min) (Figure 1). We chose these fractions because they showed the highest variability in Rt in relation to changes in eluent conditions and because these fractions were mostly present in every sample. Since the differences in the MW measurements of single fractions were not always large enough, we chose the sum of the MWs of the fraction for conclusive differences. (Figure 2(a) and (b))

Theoretically, if the calibrations hold over the ionic strength and pH eluent ranges, the MWs of the fractions and the sum of the MWs of the fractions considered should remain constant. However, this was not the case, the sum of MWs varied according to both the ionic strength and pH of the eluent (Figure 2). The MWs decreased substantially and the Rts increased with increasing eluent concentration up to 0.1 M, while the MWs remained constant at >0.1 to 0.2 M (Figure 2(a)). Similarly eluent pH variation (up to pH

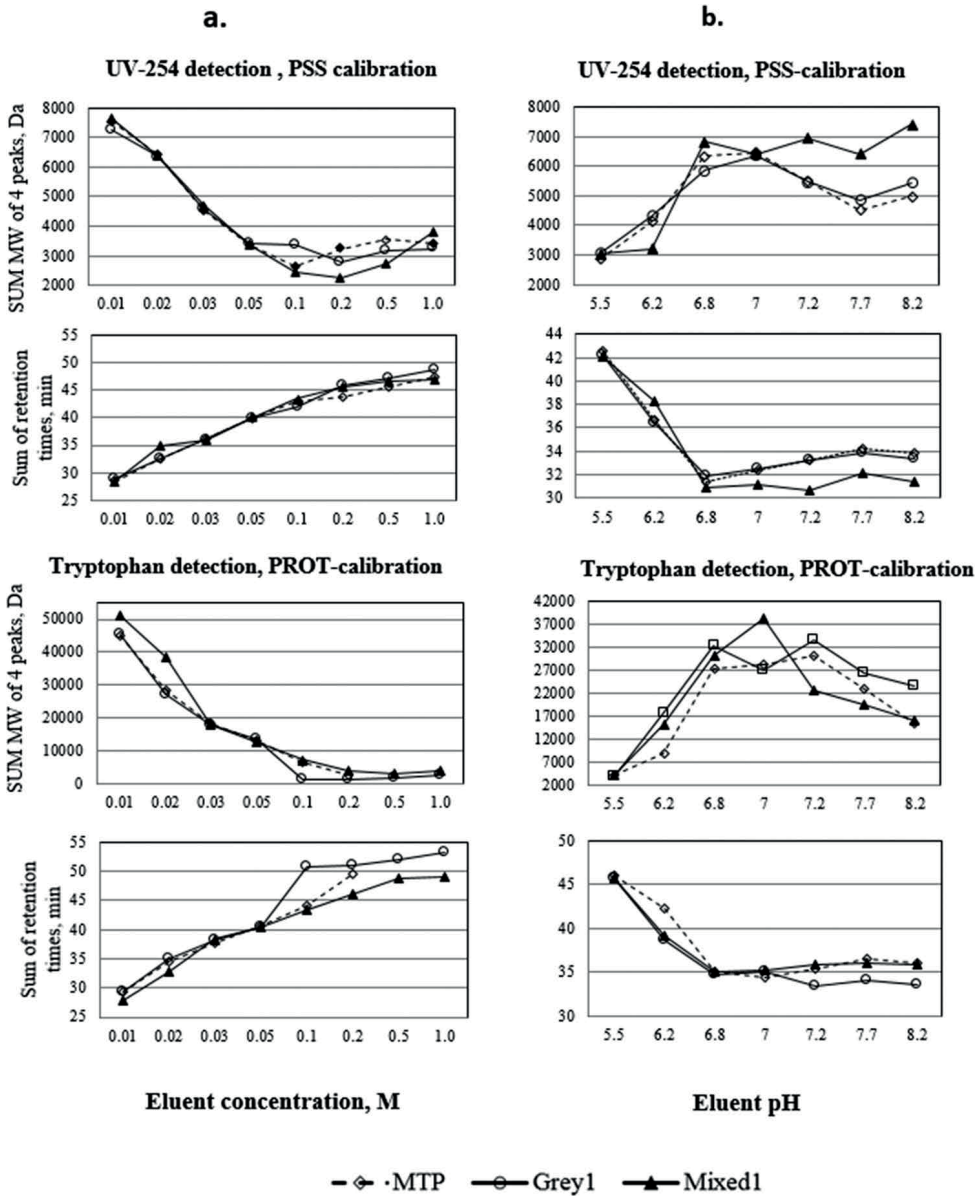

Figure 2. Sum* of MW (SUM MW) of four fractions and corresponding Rt with eluent concentration (a) and eluent pH (b) in three wastewater effluents.* Four humic-type fractions at UV-254 and the first four fractions seen by protein-type detection after 7 min. Average standard deviation: 1.3% (0–3.8%) for eluent pH runs and 0.21% (0–0.5%) for eluent concentration runs.

6.8) resulted in significant changes in the MWs, but at pH > 7, the changes were relatively less severe (Figure 2(b)).

The reasons for the MWs behaviour could be attributed to two factors: (1) the varying eluent conditions induced more changes in the size and shape of the wastewater sample components than those of the standards and (2) the secondary interactions affected more the sample molecules than those of the calibration standards. The latter is supported by the fact that R^2 values of the calibration equations decreased with increasing eluent concentration (Table 2), indicating that size exclusion becomes a less significant interaction. Literature data showed that pH variations could cause significant changes in macromolecule structure and size [16–22]. On the other hand, the higher Rt and the corresponding lower MWs at eluent pH 5.5 and 6.2 can be attributed to the weakened ion repulsion between the stationary phase and the sample molecules, with the stationary phase being charged less negatively at pH under 6.5 [1,6]. In general, pH variations affected MW measurements, but the effect was more pronounced at the acidic pH than neutral and alkaline pH (Figure 2(b)).

3.2 *The quality of separation*

The number of peaks separated and the Rt of the last peak eluted are important when assessing the size exclusion chromatograms of complex mixtures. However, during our chromatographic fractioning of the WWE, secondary interactions played an important role and there were a high number of peaks eluted over the calibration range. Hence, for correctness, we used only the term 'global resolution' to assess the quality of chromatograms, although the calculated CRF values (not shown) had similar trends. The global resolution values calculated separately for UV-254, tryptophan and tyrosine detections in three WWE are given in Table 3. The sum of the resolutions (SUM Resol) was also calculated to assess the overall chromatographic quality.

3.2.1 *Effect of injection volume*

Sample injection volume can affect resolution; too large sample volumes lead to a loss of resolution. In addition, it was previously found that there is a threshold for the injection volume (5–10% of the peak volume) and reducing it below that does not significantly improve resolution [2]. In this study, the result were mixed (Table 3): for less concentrated MTP effluent, the global resolution was higher for higher injection volumes (30 and 40 µl) and vice versa for high strength mixed effluent (best results for 10 µl injection volume), while injection volume had no significant effect on the global resolution of the greywater effluent.

3.2.2 *Effect of eluent pH*

Our results showed that eluent pH variation causes changes in the Rt, resolution and peak area of the chromatograms. The fractions influenced mostly were those eluted between 7.5 and 11.6 min Rt, and thus within the range of calibration for both UV-254 and protein-type detections (Figure 1). The fractions eluted over 15 min showed relatively constant Rt. The chromatograms from the eluent pH of 6.8 up to 8.2 had similar peaks and resolution. The sums of the resolutions are lower for the 5.5 and 6.2 eluent pH than for the higher eluent pHs tested. The resolutions of the different detection

Table 3. Global resolution of three wastewater effluent chromatograms at three different detections for different injection volumes, eluent concentrations and eluent pH values.

Wastewater effluent	MTP				Grey 1				Grey 2				Mixed 1				Mixed 2			
Detection	UV-254	Tryp*	Tyr**	SUM Resol.	UV-254	Tryp*	Tyr**	SUM Resol.	UV-254	Tryp*	Tyr**	SUM Resol.	UV-254	Tryp*	Tyr**	SUM Resol.	UV-254	Tryp*	Tyr**	SUM Resol.
Injection volume µl																				
10	3.4	2.6	0.0	6.0	6.0	***6.9***	6.2	19.1					10.5	***16.3***	***12.3***	39.0				
20	4.3	3.1	0.0	7.5	***6.9***	6.2	6.3	19.4					***10.9***	12.4	10.3	33.5				
30	***4.5***	4.1	1.8	10.3	6.4	6.3	5.3	17.9					9.9	10.6	10.8	31.4				
40	4.1	***5.1***	***1.8***	10.9	5.2	6.7	***6.7***	18.7					9.3	8.5	11.8	29.7				
Eluent concentration M																				
0.01	***6.3***	***7.7***	***3.0***	17.0	***7.4***	***7.5***	***5.1***	***20.0***	***4.8***	4.4	2.0	***11.2***	***12.6***	***15.1***	***13.6***	***41.3***	***8.7***	*12.3*	6.8	***27.8***
0.02	3.5	6.0	1.7	11.1	6.2	5.3	4.6	16.1	3.1	***5.1***	2.0	10.2	11.6	12.7	12.3	36.6	6.9	10.3	***6.9***	24.1
0.03	3.6	3.9	2.1	9.6	4.8	4.7	3.7	13.2	3.5	3.2	2.5	9.2	9.1	11.3	11.4	31.8	7.8	8.7	4.6	21.1
0.05	2.7	3.9	1.6	8.2	4.3	3.6	3.5	11.4	2.7	3.1	2.7	8.5	8.4	10.0	10.5	28.9	5.7	5.9	5.4	17.0
0.1	2.4	3.1	1.3	6.7	2.8	3.0	1.8	7.5	2.2	3.4	***3.0***	8.6	7.2	10.1	8.8	26.1	3.8	6.4	3.5	13.7
0.2	1.5	2.8	1.0	5.3	2.6	3.6	2.0	8.1	1.8	3.7	2.0	7.5	6.7	10.0	7.4	24.0	3.5	4.3	3.5	11.3
0.5	2.5	2.9	1.0	6.4	3.5	3.1	3.5	10.1	1.7	3.8	2.0	7.5	6.5	8.8	8.2	23.6	3.8	3.8	4.1	11.7
1	2.2	2.5	1.0	5.7	3.6	3.7	3.6	10.9	1.0	3.0	1.0	5.0	6.3	8.5	7.8	22.5	4.0	3.6	3.2	10.8
Eluent pH																				
5.5	2.3	1.8	1.1	5.1	2.7	4.9	***8.0***	15.5	4.2	3.4	2.7	10.4	8.4	11.6	9.4	29.4	5.3	7.6	5.4	18.3
6.2	3.2	2.9	1.3	7.4	4.5	7.0	4.4	15.9	4.1	4.1	2.5	10.7	9.9	12.5	9.8	32.2	5.3	10.7	8.1	24.1
6.8	***4.2***	***8.0***	1.9	***14.1***	5.2	7.7	3.9	16.8	4.2	***4.5***	2.7	11.4	10.3	***14.1***	11.5	35.9	7.3	10.3	7.5	25.1
7.2	4.2	4.3	1.8	10.3	***6.9***	7.7	4.7	19.4	***6.5***	***4.5***	***4.1***	***15.1***	***12.0***	13.8	12.7	***38.5***	***8.4***	***12.8***	***9.2***	***30.4***
7.7	3.5	3.6	***2.3***	9.4	6.3	8.2	5.7	20.2	5.8	***4.5***	2.4	12.7	10.8	12.1	12.8	35.7	7.3	11.7	7.7	26.7
8.2	3.6	4.3	1.9	9.7	5.6	***8.4***	7.2	***21.3***	5.9	4.3	2.8	13.0	10.8	12.8	***13.3***	37.0	***8.4***	12.6	7.0	28.0

Maximum values in bold. *Tryptophan detection; **Tyrosine detection.

chromatograms are smaller as well except for Grey 1 tyrosine and Mixed 1 tryptophan signals resolutions. The high resolution of Grey 1 tyrosine signal at pH 5.5 and at 8.2 is because of the higher peak areas at these 'extreme' eluent pH values and a 'ghost peak' at Rt = 19 min (not shown) seen only by tyrosine detection.

The slightly lower resolution values for the other then Grey 1 tyrosine signals at eluent pH 5.5 and 6.2 are due to peak compression. This can be explained by the pH influence on the quaternary, tertiary and secondary structure of proteins that might have affected the elution behaviour of large protein molecules [19,20,21,25]. Shorter Rt for higher eluent pH values had previously been observed for Nordic Fulvic acid analysed on a silicagel column and UV-254 detection [3]. These results are explained by the fact that with the increase of pH, the sample molecules became more negatively charged and consequently the repulsion between the column material and the molecules is accentuated. At high enough pH (in this study pH ≥ 6.8), the ion exclusion effect does not intensify. Three effluents had maximum resolution at eluent pH 7.2 and none of the effluents had maximum global resolution at eluent pH of 5.5 or 6.2. Therefore, we can suggest an eluent pH around neutral or slightly basic as suitable for analysis of WWE.

We assessed the effect of eluent pH on the UV absorbance and the fluorescence of the WWE by calculating the percentage variation of the total area under the chromatograms (SumPeak Area, SPA) (Figure 3(a)). The data showed that the UV-254 absorbance of WWE were consistently higher at neutral and basic eluent pH. This result is in agreement with previous findings on UV absorbance of humic matter from freshwater and isolated surface water [16–18]. Lower UV-254 absorbance at lower pH might be caused by the aggregation of protonated complex organic matter from WWE.

As expected, the results in this study showed no clear trend in the tryptophan-like fluorescence variation with eluent pH, due to the variability of the composition of the samples. These results contradict some and agree with other previous findings. According to the literature, the quantum yield of pure tryptophan is almost constant at pH values between 4 and 8 [26,27]. However, a series of protein macromolecules such as cytolytic toxin Cyt 1A, BSA, soy-glycinin [19–21], β-lactoglobulin [25], horse EE alcohol dehydrogenase and human β1β1 dehydrogenase [28] were found to have fluctuations in fluorescence intensity with pH changes. Similar behaviour was observed for freshwater organic matter [22]. Additionally, surfactants were found to quench the tryptophan fluorescence of BSA and horse serum albumin [29].

The tyrosine-like fluorescence for Grey 2 and Mixed 1 samples was relatively stable (Figure 3(a)) and the result agrees with previous findings [21,30]. On the other hand, tyrosine-like fluorescence for Grey 1 and Mixed 2 samples varied with eluent pH. Apparently, the tyrosine-like fluorescence is more dependent on the sample composition rather than on eluent pH.

3.2.3 *Effect of eluent ionic strength*

Overall, changes in the eluent ionic strength cause changes in the Rt, resolution and peak area of the chromatograms (Figure 4, Table 3). The shortest Rt and highest global resolutions were reached with the lowest eluent concentration (0.01 M), where ionic interactions predominated. Increase in eluent concentration caused increase in Rt and decrease in separation efficiency due to peak broadening, as a result of increasing hydrophobic interactions between solute molecules and column gel. UV-254

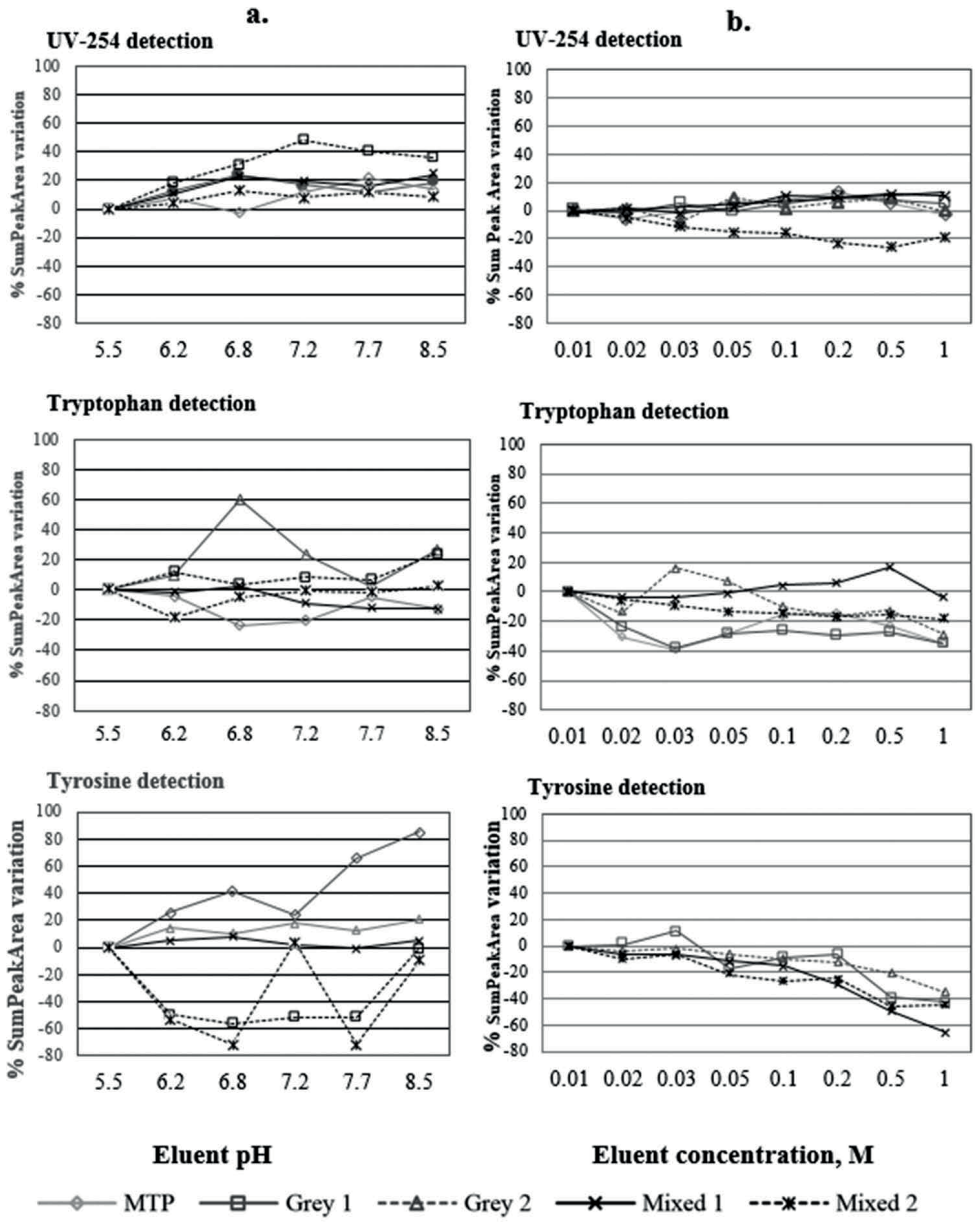

Figure 3. Variations of peak areas (SPA) with eluent pH (a) and eluent concentration (b) of five wastewater effluents at three different detections. Average standard deviation: 1.2% (0.3–2.7%) for eluent pH runs and 1.8% (0.5–4.1%) for eluent concentration runs.

chromatograms of the mixed WWEs at highest ionic strength (0.5 and 1 M) showed additional peaks eluted after 13.2 min (DNS). The UV-254 fractions eluted between 7.5 and 11.6 min, and the protein-type peaks eluted from about 7 min up to 15 min were the ones influenced most by eluent concentrations. Their Rt increased significantly (by 4–5 min) with the increase of the eluent ionic strength from 0.01 to 1 M. This behaviour was previously observed for humic-type molecules [3,6,8,11,23] as well as for protein-

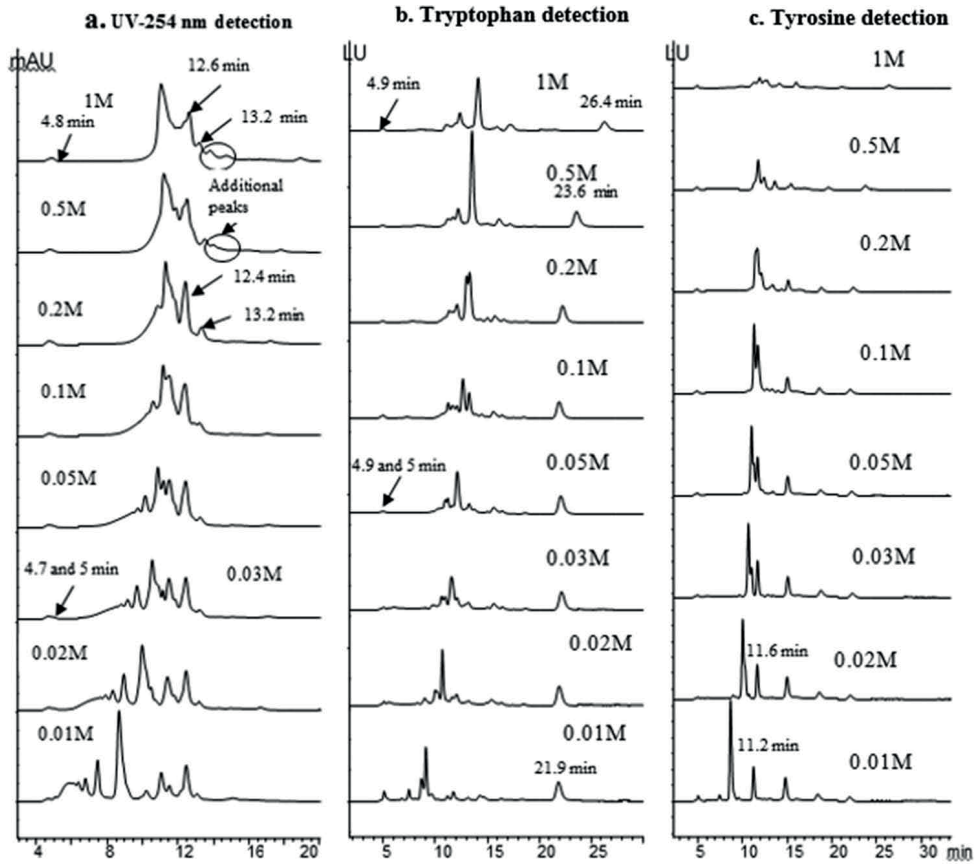

Figure 4. Chromatograms of Mixed 1 wastewater effluent at different eluent concentrations. Constant eluent pH = 7, injection volume 40 µl. mAU: milliAmpere Units; LU: Luminescence Units.

type and other biomolecules [1,2,24,31] and for simple molecules like alcohols, organic acids and amino acids [7]. For example, for humic compounds, eluent ionic strengths of 0.1 [3,11] and 0.2 M [6] were suggested as the upper limit above which hydrophobic interactions broaden the eluted peaks.

Variations in the SPA with eluent concentration are presented in Figure 3(b). The UV-254 absorbance of most of the WWEs increased slightly at eluent concentrations higher than 0.05 M, which agrees with previous studies [32,33]. Mixed 2 WWE however showed an inverse trend, possibly due to differences in composition (Table 1).

Tryptophan-like fluorescence varied with changes in the eluent ionic strength, with no clear trends – as was expected from the very few previous results found in the literature. Renard et al. [25] found a decreasing tryptophan fluorescence of β-lactoglobulin with increasing ionic strength. Another study showed that soy-glycinin had an increased tryptophan fluorescence at 0.5 M ionic strength [19].

Tyrosine-like fluorescence intensity decreased with increase in eluent concentration. Early studies have shown that tyrosine-fluorescence of the tyrosine and its related compounds decreases due to quenching caused by acetate ions [34,35]. Additionally, the Na^+ ion was also found to have a quenching effect on the tyrosine fluorescence of

some calmodulins [36]. Since these ions are components of the eluent, they can well contribute to a decrease in the total peak area of tyrosine-type peaks.

4. Conclusions

HPLC-SEC with a TSK-GEL G3000SW column and CH_3COONa eluent can be efficiently used to separate the dissolved organic components of high-strength WWE. Multiple detection using UV-254 absorption and tryptophan- and tyrosine-like fluorescence can differentiate between 'humic-type' and 'protein-type' fractions and also between tryptophan- and tyrosine-type fractions. Therefore, this simple analytical setup could be effectively used as a tool to monitor the purification performance of WWTP with respect to organic compounds.

The calibration equations determined at different eluent conditions showed that, at least with respect to the calibration standards, the size exclusion mechanism is apparently conserved up to eluent concentrations of 0.05 M and practically for all of the eluent pH values tested (5.5–8.2). However, the MWs of the WWE fractions eluted within the range of calibration cannot be accurately determined because of the secondary interactions that act on the sample molecules to a higher extent than on the calibration standards.

The global resolution was influenced by the eluent concentration, eluent pH and injection volume. The lowest eluent concentration of 0.01 M produced the best separation for all of the WWEs tested at any detection. Moreover, neutral and basic pH values of the eluent produced better separation of the fractions than acidic pH. For effluents with lower organic amounts larger injection volumes (40 µl) provide better resolution, but for more concentrated effluents, smaller injection volumes (10 µl) provide better resolution.

Regarding the variations in the UV absorbance and protein-type fluorescence, two clear tendencies were observed in this study. First, the UV-254 absorbance was clearly higher at neutral and basic eluent pH compared to the acidic eluents. Second, tyrosine-like fluorescence decreases strongly with the increase of the eluent ionic strength. No clear trend in the variation of tryptophan-like fluorescence could be observed, due to the high variety and highly variable properties of the protein type compounds normally found in the WWE.

Based on this study, we recommend low sodium acetate eluent concentrations (up to 0.03 M) at neutral or slightly basic pH for the HPLC-SEC determining DOM in WWE samples.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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PUBLICATION IV

HPLC-SEC chromatograms as surrogates for BOD and other organic quality indicators of septic tank effluents.

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ORIGINAL PAPER

HPLC-SEC chromatograms as surrogates for BOD and other organic quality indicators of septic tank effluents

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Abstract
HPLC-SEC with UV254, trytophane-like and tyrosine-like fluorescence detection was used to characterize onsite wastewater effluents from septic tanks. In total, 69 wastewater effluents, 15 of them greywater (GWE) and 54 blackwater effluents (BWE), were analysed for water quality indicators: BOD-7, DOC, COD, Total-N and TDP using conventional methods. The chromatograms showed well-separated three regions, referred to as chromatographic indicators (ChIs), which were tested as surrogates for the conventional indicators. The best surrogates for BOD-7 and DOC were found in region 3 of the tyrosine-like chromatograms. Data showed that Tyr-3, representing the sum of the tyrosine-like fractions from 8.25 min, could be used reliably to assess the BOD-7 and DOC of GWE and to approximate the BOD-7 of BWE. In addition, Tyr-3a, the fractions between 8.25 and 14 min, could be used to approximate the DOC of BWE. Furthermore, the strong correlation between COD and Tyr (all the tyrosine-type fractions) for GWE and between COD and UV254-3 for BWE allows reliable calculation of the COD of GWEs and its approximation of BWEs by linear regression. Total-N correlated weakly with tryptophan-like fractions. The use of ChI as surrogates for BOD-7, DOC and COD is an important finding that enables reliable and fast analysis without use of harmful chemicals.

Keywords Blackwater · Greywater · Fluorescence · SEC · Ionic eluent · COD · DOC

Introduction

Septic tanks are worldwide the most common systems for treating household wastewaters produced in rural areas (Eveborn et al. 2012; Withers et al. 2012; Garcia et al. 2013). The septic tank, a key purification unit for treating wastewater onsite, produces under anaerobic conditions a complex mixture of partially decomposed organic and inorganic compounds (Jenssen et al. 2010; Eveborn et al. 2012; O'Launaigh et al. 2012; Garcia et al. 2013). Faulty design and lack of routine maintenance of septic systems cause the septic tank effluent to deteriorate and thereby add to health risks and cause eutrophication of the receiving water bodies (Almomani and Khraisheh 2016). A rough estimation of an extended watershed dominated by agriculture showed that septic tank effluents contribute to as much as 14% of the total anthropogenic environmental phosphorous load (Tanik et al. 2013).

To assess the environmental loadings of organic matter and nutrients from a septic tank, fast and reliable analytical methods are necessary to characterize its effluents (STE). The conventional quality indicators used to characterize dissolved organic matter (DOM), nitrogen and phosphorous in wastewater and wastewater effluent (WWE) are 7-day biochemical oxygen demand (BOD-7), chemical oxygen demand (COD) and dissolved organic carbon (DOC) for the organic matter content, total Kjeldahl nitrogen (TKN) and total dissolved phosphorous (TDP). These indicators are all 'gross' quality indicators (sum parameters) and provide no information on particular components of organic matter. They have also other drawbacks: BOD-7 analysis is time-consuming, lasts for 7 days, and Kjeldahl and COD methods use harmful chemicals. Therefore, alternative methods are needed to replace and/or complement these conventional methods

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(Pasztor et al. 2009; Prasse et al. 2015). As we assessed in our previous study (Szabo et al. 2016), an alternative method could be aqueous, high performance, size exclusion liquid chromatography (HPLC-SEC) with multiple detection for quick information on the gross- and component organic matter content. HPLC-SEC is generally used to separate mixtures of organic compounds in water samples (biomolecules, natural organic matter) (Irvine 1997; Pelekani et al. 1999; Bouvier and Koza 2014), but it can also simultaneously be used to measure nitrate concentration in groundwater (Szabo and Tuhkanen 2010). So far, HPLC-SEC has been mainly applied to measuring the molecular weight (MW) of the fractions of organic matter mixtures, supposing a separation based solely on a size exclusion mechanism (Specht and Frimmel 2000; Figueruelo et al. 2004; Janoš and Zatrepálková 2007). However, for complex organic mixtures, such as wastewater effluents, secondary interactions occur between solute molecules and column material (Ricker and Sandoval 1996; Irvine 1997; Pelekani et al. 1999; Bouvier and Koza 2014), which interfere with correct measurements of MWs. Nevertheless, the HPLC-SEC method provides good separation and can, therefore, be used, especially with multiple detection, to characterize component organic matter: UV/VIS is suitable to detect aromatic groups present in 'humic-type' compounds and fluorescence to detect 'protein-type' compounds (Specht and Frimmel 2000; Her et al. 2003; Wu et al. 2016; Carstea et al. 2016; Szabo et al. 2016).

To ensure good separation of organic fractions in a water sample, suitable eluent at proper concentration and pH must be used. Our previous research showed that Na-acetate eluent at concentrations of up to 0.03 M with neutral or slightly basic pH can efficiently separate DOM fractions in a complex wastewater sample (Szabo et al. 2016). According to our knowledge, the use of these fractions as surrogates of the conventional organic water quality indicators of complex water samples has not been studied before. The main 'drawback' of the method is that, because of secondary interactions, it does not provide reliable MW measurements of the fractions of complex wastewater.

The main objectives of this research were (1) to characterize the organic matter of septic tank effluents by HPLC-SEC with a multiple detection method and (2) to develop a rapid and reliable HPLC-SEC method to determine surrogates for water quality indicators, mainly BOD-7, COD, DOC and TKN.

Materials and methods

Wastewater effluent samples

In total, 69 STEs were analysed. Samples were taken from small-scale, onsite wastewater systems serving single households in villages in western and southern Finland. In 15 cases, the wastewater was greywater (consisting only of household washing water because the households have dry toilets and produce no wastewater for treatment). In the remaining 54 cases, the treated wastewater was blackwater (mixed wastewater) containing both washing waters and toilet waters including urine and faeces. Greywater (GWE) and blackwater effluent (BWE) samples were taken from the supernatant (just under the surface) from the last compartment (second or third) of the septic tank or from the distribution wells after the septic tank carefully to avoid the occasional solids floating on the surface. In 13 cases, the septic tank effluents were further purified on site in constructed filtration fields, and the filtrates were collected in a manhole from where our samples were taken. Samples were collected in 1-L polyethylene bottles and kept at 5 °C until analysed within 24 h for conventional quality indicators. Each site was sampled once. For HPLC analysis, samples were quickly frozen until analysed.

Methods

Chemical analyses

The TKN of the samples was measured according to the Kjeldahl method (SFS 5505), whereas total dissolved phosphorous (TDP) was determined spectrophotometrically (SFS 3026). The organic content of the samples was measured as BOD-7, DOC and COD. BOD-7 was measured using the OxiTop OC 100 WTW system with an accuracy of ± 1% of the measured value and ± 7% of the measuring range. The samples were not diluted, because the measuring range (500–1350 hPa) was not exceeded. To prevent nitrification, allylthioureaa (ATU) was added to each sample before they were incubated at 20 °C for 7 days. BOD-7 values were directly read from the instrument.

COD was determined according to the Finnish Standard SFS 5504, using Hach Lange LT 200 Dry Thermostat, UK, for digestion followed by titration according to the method specifications. DOC was measured as non-purgeable organic carbon with a TOC analyser, the SHIMADZU TOC-5000. Before DOC analysis, the samples were filtered through a 0.45-µm filter (Whatman).

HPLC-SEC analyses

HPLC-SEC analysis was done with a Hewlett-Packard HPLC 1100 system, using a TSK-GEL G3000SW 7.5 mm × 30 cm column (TOSOH Bioscience). Two detectors were used in tandem: a UV/VIS HP 1100 Series Diode Array Detector (detection wavelength 254 nm (UV254)) and an HP 1100 Series Fluorescence Detector with two excitation/emission wavelengths set for tryptophan-like detection (Ex/Em 270 nm/355 nm) and tyrosine-like detection (with

Ex/Em 270/310). The eluent used was 0.02 M Na-acetate with pH 7 and a flow rate of 1 mL/min. The sample injection volume was 20 µL with elution time set to 35 min. The conditions produced good global resolution for both GWE and BWE (Szabo et al. 2016). Before analysis, the samples were filtered with a 0.45-µm filter (Whatman). For each sample injection, three chromatograms were obtained, integrated manually with their peak areas used to assess organic matter in the samples. The void volume of the column (4.6 min) was determined with Blue Dextrane (molecular weight MW = 10^6 Da, FLUKA, Switzerland), the permeation volume for UV-signals (11.6 min) was measured with acetone (MW = 58 Da), and the permeation volume for tryptophan- and tyrosine-like signals (12.4 min) was measured with tryptophan, as reported previously (Szabo et al. 2016). However, we did not calibrate the column to determine molecular weight, since secondary interactions strongly affect retention times and make molecular weight calculations irrelevant (Szabo et al. 2016).

Statistical analyses

The goodness of fit to the normal distribution of data was tested with the one-sample Kolmogorov–Smirnov test. Since only 6 out of 23 examined parameters showed normal distribution, Spearman's rank correlation coefficient was used to evaluate the degree of association between the parameters. Statistical analysis was performed with the statistical program SPSS version 23 (IBM). Linear regression between correlating data was done with Excel 2016.

Results and discussion

Quality of septic tanks effluent

The mean, maximum and minimum values of the conventional quality indicators of onsite wastewater effluents are given in Table 1. The values present great variety within BWE and within GWE samples, depending obviously on the number and customs of the septic tank users as well as the characteristics of the onsite system.

Our TKN (107.9 mg/L) and COD (661.9 mg/L) mean values for BWE are higher than those found in the literature (Corbett et al. 2002; Jenssen et al. 2010; O'Launaigh et al. 2012, respectively). On the other hand, our results for TDP (11.3 mg/L) and BOD (269.7 mg/L) correspond with those reported in the literature (Corbett et al. 2002; Jenssen et al. 2010; O'Luanaigh et al. 2012). As expected, a clear difference can be seen between the qualities of the two types of effluents. However, the mean GWE values measured in this study (Table 1) are higher than those reported earlier in one comparative study (Brandes 1978), in which total phosphorus (TP) values of 1.4 mg/L and TKN values of 11.3 mg/L were measured in a GWE, values that were 10 times lower than the corresponding values in BWE. In our study, GWE mean values for all conventional indicators were lower than BWE means: GWE means are 24% of TDP-, 24% of TKN-, 66% of BOD-7-, 59% of COD- and 66% of DOC-BWE means (Table 1). Because environmental loading depends also on the volume of the effluents discharged, high effluent concentrations do not necessarily mean high loading. In addition to effluent concentrations, correct assessment of the loading from septic effluents must take into account the number of persons using the septic system and their water consumption. The mean biodegradability of GWE and BWE assessed as BOD-7/COD was > 0.4 (0.41 for BWE and 0.46 for GWE), which indicates biodegradable effluents (Chamarro et al. 2001), that is, effluents that will further degrade once released into the environment.

Sample analysis by HPLC-SEC

Quantitative analysis

Typical chromatograms of BWE and GWE are given in Fig. 1. The chromatograms have several and different peaks with different types of detection and show diverse

Table 1 Characteristics of blackwater effluents BWE (a) and greywater effluents GWE (b)

	a					b				
	#	Minimum	Maximum	Mean	SD	#	Minimum	Maximum	Mean	SD
pH	54.0	5.6	8.8	7.2	0.6	15.0	5.9	7.9	6.8	0.5
TDP (mg/L)	54.0	0.4	31.0	11.3	6.1	15.0	0.2	12.7	2.7	3.2
TKN (mg/L)	54.0	6.0	390.6	107.9	76.3	15.0	0.4	224.0	26.5	56.0
BOD-7 (mg/L)	54.0	5.6	543.0	269.7	150.1	14.0	14.1	509.0	177.1	156.6
COD(mg/L)	41.0	41.0	2853.0	661.9	498.5	11.0	35.0	980.0	389.8	286.8
DOC (mg/L)	54.0	5.3	246.0	91.8	58.9	15.0	5.1	244.0	60.9	63.1

TDP total dissolved phosphorous; *TKN* total Kjeldahl nitrogen; *BOD-7* biochemical oxygen demand; *COD* chemical oxygen demand; *DOC* total dissolved organic carbon

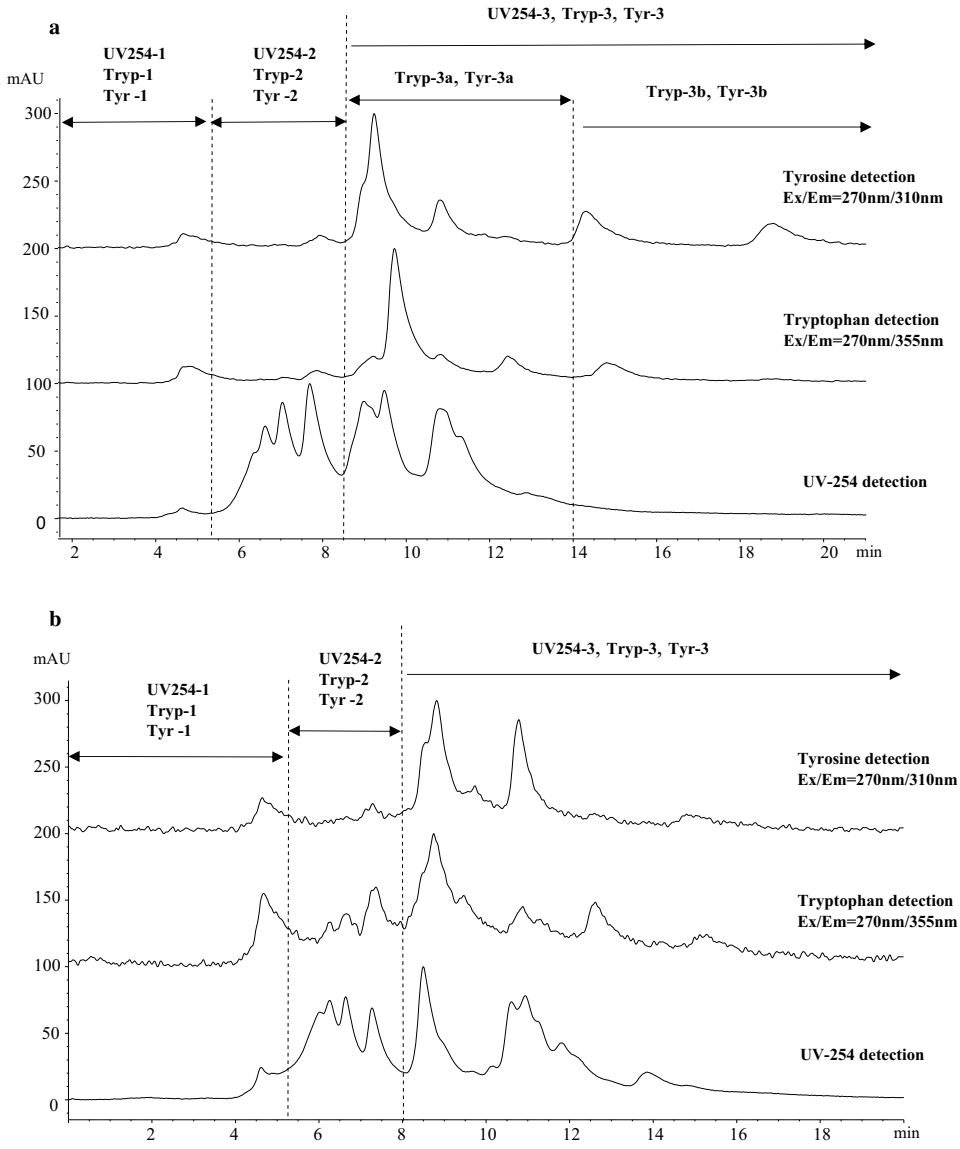

Fig. 1 Chromatograms at different detections of BWE (**a**) and GWE (**b**). Eluent: 0.02 M CH_3COONa, pH: 7.2, mAU (milliAmpere Units). Effluent characteristics: BWE: TKN = 164 mg/L, BOD-7 = 543 mg/L, COD = 1500 mg/L, DOC = 170 mg/L; GWE: TKN = 35.4 mg/L, BOD-7 = 509 mg/L, COD = 980 mg/L, DOC = 244 mg/L

and numerous molecules. The chromatograms are complex because of numerous organic molecules and extracellular polymers excreted by viable cells in the wastewater, a finding that agrees with a previous study on extrapolymeric substances extracted from anaerobic sludge (Bahtia et al. 2013). We divided the chromatograms into three regions: region 1 of fractions eluted near a void volume of 4.5 mL, region 2 of fractions eluted between 5 min and 8.25 min, and region 3 of fractions eluted after 8.25 min. (Figure 1). For BWE samples, region 3 fluorescence signals

were divided into two additional sub-regions: region 3a of peaks between 8.25 min and 14 min and region 3b of peaks eluted after 14 min. (Figure 1). The chromatograms were divided into the above three regions because region 1 peaks were distinct from region 2 peaks and were probably influenced by secondary, repulsive interactions (Ricker and Sandoval 1996; Pelekani et al. 1999; Bouvier and Koza 2014). Region 2 peaks are regularly observed in natural waters, such as surface and ground waters, and are considered 'humic' compounds (Peuravuori and Pihlaja 1997; Szabo and Tuhkanen 2010). Region 3 peaks are not observed in natural water samples but are seen in complex wastewater effluents, and their late elution is probably due to secondary attractive interactions within the column (Szabo et al. 2016). Sub-region 3b peaks (one or two peaks) were detected only by fluorescence detection in BWE, and by separating the peaks, we tried to determine their nature.

The ChrInds are peak areas expressed in mAU*min of different regions of different detections. In this way, 13 'individual' ChrInds could be distinguished: UV254-1, UV254-2, UV254-3, Tryptophan-1 (Tryp-1), Tryp-2, Tryp-3, Tryp-3a, Tryp-3b, Tyrosine-1 (Tyr-1), Tyr-2, Tyr-3, Tyr-3a, Tyr-3b. In addition, three 'sum-ChrInds' were calculated by summing up all the peaks of the corresponding detection, respectively, UV254, Tryp and Tyr.

The minimum, maximum and mean values of the different ChrInds are given in Table 2. As expected, GWE samples had lower ChrInd values than BWE samples. The mean values of GWE were from 8.4% (Tyr-1) to 62.9% (Tryp-2) of those of BWE values.

The data on BWE and GWE differ because of their different compositions (BWE contains additional faeces, urine and toilet paper), and as such are not directly comparable. Therefore, we calculated the Spearman rank correlation coefficients between ChrInd and conventional indicators separately for BWE and GWE (Table 3).

Table 3 shows that the organic matter indicators of the effluents (BOD-7, COD and DOC) correlate significantly with all ChrInds, except for GWE regions 1 and 2. Region 1 fractions were eluted near the void volume of 4.6 min and, according to SEC theory, were influenced by repulsive electrostatic interactions or had large molecular weight, around 10^6 Da (Irvine 1997; Ricker and Sandoval 1996; Pelekani et al. 1999; Specht and Frimmel 2000). The conventional indicators and region 1 ChrInd for GWE correlated poorly because region 1 peaks were absent in 8 (53%) GWE samples but only in 5 (9%) BWE samples. Region 2 showed 'humic-type' fractions within the calibration range, which are naturally present in waters bodies, and are traditionally detected by UV254 (Peuravuori and Pihlaja 1997; Specht and Frimmel 2000; Szabo et al. 2008). In our study, among the three detections used, UV254 was also the best in detecting region 2 peaks. UV254 region 2 fractions were present in all of the analysed WWE samples, whereas fluorescence detected mostly the lowest molecular weight fractions in region 2. Fractions eluted at around 7.6 min represent humic molecules, inorganic nitrate (Szabo and Tuhkanen

Table 2 Chromatographic indicators (ChrInd) of mixed (a) and grey (b) wastewater effluents

Chromatographic indicators ChrInd (mAU*min)	a					b				
	#	Minimum	Maximum	Mean	SD	#	Minimum	Maximum	Mean	SD
UV-254	54.0	23.8	2008.9	725.4	380.2	15.0	101.6	993.3	281.5	214.0
Tryp	54.0	8.8	1053.3	362.3	251.0	15.0	2.0	263.3	89.6	75.3
Tyr	54.0	0.0	1066.9	350.5	272.3	15.0	1.0	249.1	60.5	63.1
UV254-1	54.0	0.0	47.2	14.3	10.4	15.0	0.0	36.0	3.5	9.1
UV254-2	54.0	15.5	1075.9	319.1	179.1	15.0	27.0	379.7	130.3	83.3
UV254-3	54.0	8.3	995.1	391.8	233.5	15.0	17.0	577.6	147.6	136.6
Tryp-1	54.0	0.0	117.0	29.0	22.6	15.0	0.0	35.4	5.7	9.4
Tryp-2	54.0	0.0	114.9	37.9	21.3	15.0	0.0	168.0	23.8	42.0
Tryp-3	54.0	8.8	905.6	294.9	218.2	15.0	0.0	173.2	60.0	48.8
Tryp-3a	54.0	8.8	815.3	276.8	202.0	15.0	0.0	0.0	0.0	0.0
Tryp-3b	54.0	0.0	90.3	18.1	23.1	15.0	0.0	0.0	0.0	0.0
Tyr-1	54.0	0.0	70.8	19.8	14.3	15.0	0.0	9.6	1.7	3.1
Tyr-2	54.0	0.0	116.8	20.4	17.3	15.0	0.0	11.3	2.9	3.4
Tyr-3	54.0	0.0	1038.7	315.2	253.4	15.0	0.0	203.2	52.9	54.2
Tyr-3a	54.0	0.0	852.3	226.9	185.8	15.0	0.0	0.0	0.0	0.0
Tyr-3b	53.0	0.0	330.2	85.5	93.6	15.0	0.0	0.0	0.0	0.0

Table 3 Spearman rank correlation coefficients between chromatographic and other indicators for mixed (a) and grey (b) wastewater effluents

Chromatographic indicators ChrInd (mAU*min)	a					b				
	TDP (mg/L)	TKN (mg/L)	BOD-7 (mg/L)	COD (mg/L)	DOC (mg/L)	TDP (mg/L)	TKN (mg/L)	BOD-7 (mg/L)	COD (mg/L)	DOC (mg/L)
UV254	0.338*	0.641**	0.558**	0.680**	0.750**	0.252	0.459	0.873**	0.873**	0.863**
Tryp	0.496**	**0.665****	0.689**	0.632**	0.818**	−0.195	**0.622***	0.788**	0.818**	0.817**
Tyr	0.437**	0.518**	0.761**	0.631**	0.870**	0.259	0.356	0.959**	**0.973****	0.945**
UV254-1	0.274*	0.15	0.438**	0.425**	0.598**	−0.177	0.575*	0.378	0.411	0.469
UV254-2	0.298*	0.556**	0.331*	0.525**	0.482**	0.131	0.300	0.196	0.227	0.157
UV254-3	0.332*	0.627**	0.668**	**0.697****	0.851**	0.145	0.461	0.937**	0.909**	0.949**
Tryp-1	0.391**	0.361**	0.558**	0.572**	0.666**	0.062	0.563*	0.486	0.560	0.498
Tryp-2	0.332*	0.636**	0.517**	0.697**	0.672**	0.205	0.066	0.632*	0.400	0.446
Tryp-3	0.508**	0.657**	0.708**	0.640**	0.826**	−0.111	0.622*	0.840**	0.847**	0.895**
Tryp-3a	0.485**	**0.661****	0.705**	0.639**	0.832**	No	No	No	No	No
Tryp-3b	0.446**	0.342*	0.539**	0.502**	0.577**	No	No	No	No	No
Tyr-1	0.411**	0.388**	0.521**	0.552**	0.637**	0.073	0.307	0.215	0.350	0.225
Tyr-2	0.280*	0.465**	0.296*	0.338*	0.432**	0.102	−0.537	−0.153	0.064	−0.129
Tyr-3	0.424**	0.479**	**0.791****	0.624**	0.887**	0.235	0.465	**0.968****	0.929**	**0.951****
Tyr-3a	0.308*	0.460**	0.768**	0.636**	**0.893****	No	No	No	No	No
Tyr-3b	**0.563****	0.320*	0.612**	0.497**	0.628**	No	No	No	No	No

The highest values are in bold type

*Correlation is significant at 0.05 level (2-tailed)

**Correlation is significant at 0.01 level (2-tailed)

2010), and some organic molecules with tryptophan-like and tyrosine-like fluorophores (Fig. 1).

For region 2, tyrosine signals were weaker than tryptophan signals, especially for GWE with 7 (47%) samples having no tyrosine fraction in this region. This explains the non-significant correlations between Tyr-2 and conventional organic indicators for GWE. For BWE region 2 ChrInd, UV254-2, Tryp-2 and Tyr-2 fractions correlated better with COD and DOC than with BOD because of the relatively poor biodegradability of the humic matter (Leenheer and Croué 2003; Marschner and Kalbitz 2003).

Region 3 covered fractions UV254-3, Tryp-3, Tryp-3a, Tryp-3b, Tyr-3, Tyr-3a and Tyr-3b. These fractions, eluted after the permeation volume, are influenced, according to SEC theory, by secondary interactions: strong ionic or strong hydrophobic attraction between column material and fraction molecules (Irvine 1997; Ricker and Sandoval 1996; Pelekani et al. 1999; Specht and Frimmel 2000; Janoš and Zatrepálková 2007). Correlating significantly and strongly with the conventional organic matter indicators (Table 3), the fractions of region 3 are potentially the best surrogates for organic matter indicators.

BOD-7 and DOC correlate surprisingly strongly with Tryp-3, Tryp-3a, Tyr-3 and Tyr-3a fractions for both types of effluents, showing that these fractions comprise biodegradable compounds, which are also reliably measured by DOC. Similar results have been reported on tyrosine- and tryptophan-type components of the excitation–emission matrix of municipal wastewater, sampled at different purification phases and correlating best with DOC (Yu et al. 2014). The slightly stronger correlation coefficients of DOC over those of BOD-7 are possible because BOD-7 depends on the microbial diversity of the sample, which varies from sample to sample and affects BOD-7 values (Jouanneau et al. 2014).

For BWE, COD showed a lower correlation coefficient for region 3 fractions Tryp-3 and Tyr-3 than BOD-7 and DOC and a consistent correlation with all the chromatographic fractions, probably because of oxidant consumption during COD measurement, which reduced inorganic compounds in the anaerobic effluents not detectable by HPLC-SEC. One such reducing component is the reduced form of N (NH_3/NH_4^+), which originates from urine (Udert et al. 2003). For GWE, all BOD-7, COD, and DOC correlated strongly with region 3 fractions in all detections, showing that these effluents contain less reducing inorganic compounds than BWE effluents, which is obvious since greywater effluents contain no urine.

TKN (Kjeldahl) correlated significantly with all ChrInds, except for one (UV254-1) for BWE. For GWE, there were only four significant correlations (Tryp, UV254-1, Tryp-1 and Tryp-3) (Table 2). In general, TKN correlated better with tryptophan-like fractions (Tryp, Tryp-1, Tryp-2, Tryp-3 and Tryp-3a), probably because the tryptophan molecule, a part of the compounds detected in tryptophan detection, contains two N atoms, whereas the tyrosine molecule contains only one N atom. The sum-ChrInd (UV254, Tryp and Tyr) correlated better than 'individual' ones, suggesting that N is present in all fractions. In addition, the uncertainties related to TKN measurements by the Kjeldahl method may contribute to relatively weak correlations.

TDP correlated significantly but weakly with all the ChrInd of BWE and insignificantly with any ChrInd of GWE (Table 3), probably because of the low amount of TDP left in GWE (Table 1). Interestingly, for BWE, TDP correlated most strongly with Tyr-3b, which represents the last two fractions eluted after 14 min for the BWE samples (Fig. 1a). This suggests that these fractions contain most of the dissolved phosphorous left in BWE. Moreover, the interaction between the column material and these late eluted peaks may be strongly hydrophobic, as supported by a previous study, which found that most dissolved organic phosphorous belonged to the hydrophobic fraction of the organic matter dissolved in wastewater effluents (Qin et al. 2015).

Linear regression

To assess the quantitative relationship between conventional indicators and ChrInd, linear regression was done on a particular conventional indicator and the corresponding ChrInd with which it correlated the strongest (Fig. 2, bold in Table 3).

Because regression showed high R^2 values for greywater effluent BOD-7-Tyr-3, DOC-Tyr-3 and COD-Tyr, the equations can be used to reliably assess BOD-7, DOC and COD values of greywater effluents from Tyr-3 and Tyr data. For BWE, we excluded one outlier for BOD-7 and two outliers for COD, thereby gaining increased R^2 values for the corresponding regression equations and allowing assessment of approximate values of BOD-7, DOC, and COD of BWE from Tyr-3, Tyr-3a, and UV-3 data.

TKN showed weaker correlations with ChrInd than with the conventional organic indicators. However, the regression equations between TKN-Tryp gained by excluding two outliers from greywater effluents and four outliers from BWE can be used to approximate TKN values from Tryp data.

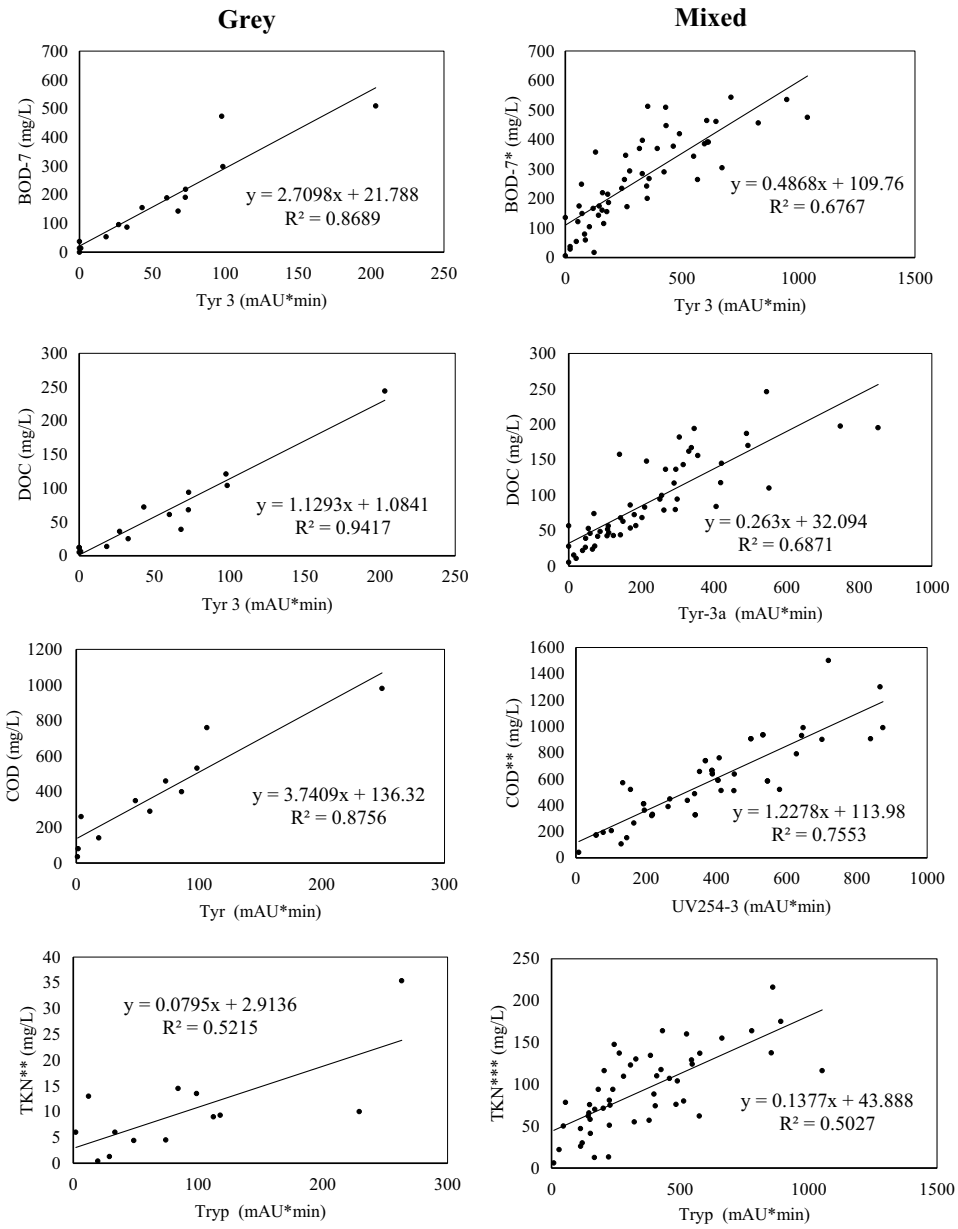

Fig. 2 Linear regression between conventional organic quality indicators and chromatographic fractions with which they best correlate. *One outlier excluded from regression; **two outliers excluded from regression; ***four outliers excluded from regression

Conclusion

Besides the current practice of measuring MW, our results show that HPLC-SEC can also be used to assess BOD and other conventional organic matter indicators (COD, DOC) of wastewater effluents. The TSK-GEL G3000SW silica gel column and Na-acetate eluent 0.02 M provided good separation of dissolved organic matter components from complex and less complex onsite wastewater effluents. The most important advantage of this method is that it gives us insight into

the composition of DOM by differentiating the major organic components in water samples as fractions rather than as sum parameters given by COD, BOD and DOC. Additional advantages are short analysis time and no use of harmful chemicals.

A large part of wastewater fractions was eluted over the permeation volume, where secondary interactions with the column material predominated. The last two fractions, seen only in BWE, eluted at around 15 min and 19 min, were probably affected by strong attractive hydrophobic interaction. Multiple detection with UV254 absorption and tryptophan- and tyrosine-like fluorescence showed well-separated regions in the chromatograms, which correlated with the conventional water quality indicators and could be used as their surrogates.

In the chromatograms, region 3 or parts of it (Tyr-3, Tyr-3a and UV254-3) described best the organic matter indicators (BOD-7, DOC and COD). The BOD-7 values of BWE and GWE correlated best with Tyr-3, that is, the sum of the fractions eluted after 8.25 min.

The regressions obtained from our data allow reliable assessment of the BOD-7 of GWE and approximation of the BOD-7 of BWE. This is an important finding, because an HPLC-SEC run of one sample requires only a 1/2 h as opposed to BOD-7 lasting for 7 days. DOC showed the best correlation with Tyr-3 for GWE and with Tyr-3a of fractions between 8.25 and 14 min for BWE. On the other hand, COD correlated best with Tyr (all tyrosine-type fractions) for GWE and with UV254-3 for BWE. The corresponding regression equations can be used to reliably calculate DOC and COD for GWE and to approximate them for BWE.

TKN correlated best with tryptophan-like fractions, but regression gave an R^2 value of 0.52 only for both types of wastewater, showing that Tryp (sum of all tryptophan-like fractions) could be used only to roughly estimate the TKN value of wastewater effluents.

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