**The influence of salinity on the molecular and optical properties of surface microlayers in a karstic estuary**

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Abstract

Sea-surface microlayers and the corresponding underlying waters of the karstic Krka Estuary (Croatia) were studied with respect to optical and molecular properties of dissolved organic matter (DOM). Solid-phase extracted DOM was separated by reversed-phase chromatography and analyzed with ultra-high resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS). The number and summed magnitudes of FT-ICR MS peaks, enriched in the microlayer, increased with increasing salinity along the estuary. The molecular hydrogen to carbon ratio (as a measure of polarity) of enriched compounds was higher for the low salinity samples than for a high salinity marine station, which we propose is a consequence of a salt-mediated separation mechanism. Absorption and fluorescence of all samples decreased along the estuary with the microlayer samples showing higher absorption than the underlying water. Chromatographic and FT-ICR MS data revealed a distinct shift towards a smaller molecular size in the microlayer compared to the underlying water. The redistribution of dissolved organic carbon within chromatographic fractions and the decrease in molecular size was interpreted to result from photo-degradation and/or microbial reprocessing. Collision induced dissociation of selected FT‑ICR MS mass peaks revealed the presence of sulfur containing anthropogenic surfactants enriched in the microlayer. Molecular level investigation of estuarine surface microlayers will help to better understand the highly dynamic character of these systems, the accumulation of natural organic matter and anthropogenic pollutants and the role of surface microlayers for the sea-air energy exchange.

Introduction

The surface microlayer is the phase-boundary between hydrosphere and atmosphere of aquatic systems (lakes, rivers, estuaries and oceans). Surface films are microhabitats, photochemical reactors, filters and physical membranes and control the gas exchange between air and water ([Liss and Duce, 1997](#_ENREF_53)). Various definitions exist for the microlayer, mainly defined operationally according to the sampling technique (glass plate, Garrett’s screen, rotating drum) and the study subject (biological, chemical or physical properties). The vertical extent of the microlayer ranges from molecular monolayers up to millimeters ([Hardy, 1982](#_ENREF_29)). A visual representation of a coherent organic film at the sea surface is the sea slick, which appears quickly during calm wind conditions ([Hunter and Liss, 1981](#_ENREF_33)). Sea-surface microlayers (SML) are known to exist even at higher than global average wind speeds, potentially covering most of the ocean’s surface at any time, hence they are of global importance ([Wurl et al., 2011](#_ENREF_78)).

The SML consists generally of adsorbed surface active substances (SAS) that are amphiphilic molecules reducing the surface tension of the water-air interface. This includes a large variety of substances such as polysaccharides ([Sieburth et al., 1976](#_ENREF_67)), transparent exopolymer particles (TEP, [Wurl and Holmes, 2008](#_ENREF_75)), polypeptides ([Kuznetsova et al., 2004](#_ENREF_47)), lipid-like material ([Gašparović et al., 1998](#_ENREF_24); [Kattner and Brockmann, 1978](#_ENREF_38); [Lass and Friedrichs, 2011](#_ENREF_48)) but also living bacteria ([Cunliffe et al., 2011](#_ENREF_15)) and phytoplankton ([Hardy and Apts, 1984](#_ENREF_30); [Joux et al., 2006](#_ENREF_35)) and their exudates (e.g. [Kattner et al., 1985](#_ENREF_40)). Many studies demonstrated that hydrophobic substances accumulate in the SML (e.g. [Kattner et al., 1983](#_ENREF_39)), including anthropogenic pollutants such as hydrocarbons and trace metals ([Guitart et al., 2007](#_ENREF_28); [Wurl and Obbard, 2004](#_ENREF_77)). The chemical composition of the SML and the enrichment factors of individual substances vary widely in time and space, depending on the trophic state of the system, wind regime, seasonality and anthropogenic pollution. Differences in the composition of the SML and the underlying subsurface water are reflected in the dissolved organic matter (DOM) composition, e.g. amino acid concentrations ([Kuznetsova et al., 2004](#_ENREF_47)), chromophoric dissolved organic matter (CDOM) distribution ([Tilstone et al., 2010](#_ENREF_71)), physico-chemical properties ([Zhang et al., 2003](#_ENREF_82)) and bacterial production and respiration ([Reinthaler et al., 2008](#_ENREF_61)). However, an exact description of the SML lacks detailed and comprehensive molecular information on the identity of the constituting substances, although some recent studies report high resolution mass spectrometry data ([Frew et al., 2006](#_ENREF_21); [Morales-Cid et al., 2009](#_ENREF_57); [Schmitt-Kopplin et al., 2012](#_ENREF_64)).

For the chemical characterization of natural organic matter (NOM) salt-free and pre-concentrated extracts are obtained by e.g. solid-phase extraction ([Dittmar et al., 2008](#_ENREF_18)). This is a prerequisite for many analytical techniques, such as reversed-phase chromatography, nuclear magnetic resonance spectroscopy or mass spectrometry. Ultra-high resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) is an advanced analytical tool to study the extremely complex mixtures of NOM ([Hertkorn et al., 2008](#_ENREF_31); [Xian et al., 2012](#_ENREF_79)). Due to its high mass resolution and accuracy, several thousands of molecular formulas per sample can be identified. Recent applications to investigate DOM include soil porewater ([D'Andrilli et al., 2010a](#_ENREF_16)), groundwater ([Longnecker and Kujawinski, 2011](#_ENREF_55)), river and coastal water ([Liu et al., 2011](#_ENREF_54); [Stubbins et al., 2010](#_ENREF_68)), open ocean and deep sea water ([D'Andrilli et al., 2010b](#_ENREF_17); [Flerus et al., 2012](#_ENREF_20)), as well as sediment porewater ([Schmidt et al., 2009](#_ENREF_63)). The information derived by FT-ICR MS is typically restricted to elemental composition and is non-quantitative. However, recent improvements of this technique included single mass fragmentation capabilities which lead to structural information on individual NOM molecules ([Witt et al., 2009](#_ENREF_74)). By combining reversed-phase chromatographic separation with FT-ICR MS the molecular complexity of NOM samples can be reduced, which enhances the analytical resolving power and enables to chemically characterize sample moieties ([Koch et al., 2008](#_ENREF_43); [Liu et al., 2011](#_ENREF_54)).

The Krka Estuary is particularly suitable to identify the chemical characteristics of the organic surface microlayer since constantly low terrestrial discharge results in relatively homogeneous organo-chemical conditions ([Cauwet, 1991](#_ENREF_10)). Therefore extraction and analytical biases due to the different chemical composition of samples along the estuary are potentially minimized. On the other hand, the strong horizontal salinity gradient und vertical stratification of the Krka Estuary allows studying microlayer versus underlying water composition under different ionic strength conditions and seasonal variations.

In our study, we present the first detailed chemical description of sea-surface microlayers compared with subsurface water with advanced molecular methods. Samples from the Krka Estuary were analyzed with FT-ICR MS and reversed-phase high performance liquid chromatography (RP-HPLC) using absorption and fluorescence detection. This combination of methods was applied to identify so far chemically uncharacterized components of the microlayer and to study their distribution patterns. We aimed to derive insights into the molecular properties of the microlayer and the bulk subsurface water and the influence of increasing salt concentration on the chemical properties of microlayer-enriched substances. This molecular information will help to better describe the chemical processes leading to a phase transfer from underlying water into the microlayer.

Methods

Sampling site and sample collection

Samples were collected in the highly stratified estuary of the Krka River in the Middle Eastern Adriatic Sea near the Croatian city of Šibenik. The hydrographic and biological features of this estuary are described in detail elsewhere ([Legović et al., 1994](#_ENREF_51); [Svensen et al., 2007](#_ENREF_69); [Vojvodić and Ćosović, 1992](#_ENREF_72)). For reasons of comparability we adopted station labels from these previous studies.

Surface microlayer (SML) and underlying water (ULW) samples were collected at three stations: In the upper estuary (Lake Prokljan, E3), characterized by low salinity and low anthropogenic impact ([Vojvodić and Ćosović, 1992](#_ENREF_72)), in the lower estuary, near the city of Šibenik (E4a) with the only significant anthropogenic influence on the estuary ([Legović et al., 1994](#_ENREF_51)), and in the saline waters off the coast of Zlarin Island (C1). Samples were collected on September 9, 2008 (E4a only) and between May 18 and May 20, 2009 (E3, E4a and C1). It should be noted that the marine station C1 is located 4 km away from the mouth of the estuary, therefore only partly reflecting a parameter continuum. However, this station has been extensively used as reference station in the past.

SML samples (0.5‑1 L) were derived with a Garrett-type screen made of stainless steel, with 1.03 mm2 mesh size and 0.24 mm wire diameter and poured into a clean glass bottle. The screen was cleaned with dichloromethane and rinsed with sample water prior collection and glass bottles were cleaned with chromosulfuric acid and thoroughly rinsed with ultra pure water. The thickness of the sampled water layer was determined from the collected water volume being approximately 110 μm. ULW (4 L) was collected directly with a clean glass bottle from 0.4 m depth. To avoid surface film contamination, the sampling bottle was slowly pushed underwater and opened at depth. All samples were GF/F filtered (~0.7 µm nominal pore size, Whatman). Samples for nutrient analysis were poisoned and stored at 4 °C ([Kattner, 1999](#_ENREF_36)). Acidified samples (pH 2 with hydrochloric acid; suprapur, Merck) for DOC determinations were stored frozen at ‑20 °C and solid-phase extraction (SPE, 1 g; Mega Bond Elut, PPL, Varian) was performed according to Dittmar et al. ([2008](#_ENREF_18)). Briefly, methanol (3 mL) and acidified (pH 2 with hydrochloric acid; suprapur) ultra pure water (3 mL) were used the clean and pre-condition the adsorbent. After applying the samples, remaining salt was rinsed with 3 mL acidified ultra-pure water and the cartridge bed dried with a N2- flow. The eluted SPE samples (3 ‑ 5 mL methanol, LichroSolv, Merck) with a nominal enrichment factor between 200 and 900 were stored at -20 °C until FT-ICR MS and RP-HPLC analysis.

DOC and nutrient analysis

DOC was determined by high temperature catalytic oxidation (TOC‑VCPN analyzer, Shimadzu). For external calibration potassium hydrogen phthalate (KHP, Merck) was used. Aliquots of the methanol extracts (50 µL) from the SPE samples were evaporated under N2 gas flow to complete dryness and subsequently redissolved in 6.5 mL ultrapure water for DOC analysis (SPE-DOC). All samples (in duplicate) were acidified (0.1 M HCL suprapur, Merck) and purged with O2 for > 5 min. Performance of the instrument was recorded by daily analysis of in-lab KHP standard solutions and reference samples (deep sea reference, DSR, Hansell research lab). The average instrument blank was 3.4 µM C (*n* = 11) and repeatability of the DSR was > 95%. The SPE efficiency [%] was calculated as 100 × SPE-DOC [µM] / (enrichment factor × DOC [µM]). Nutrient samples (nitrate, nitrite, phosphate, silicate) from the 2009 campaign were analyzed using an autoanalyzer (Evolution III, Alliance instruments) with standard seawater methods ([Kattner and Becker, 1991](#_ENREF_37) and references therein).

HPLC measurements

An HPLC system (Hitachi/VWR) was used for the chromatographic separation of the SPE samples ([Koch et al., 2008](#_ENREF_43)). The system consisted of a gradient pump (L‑2130), autosampler (L‑2200), column oven (L‑2300), diode array detector (DAD, L‑2450, optical path length: 1 cm) and fluorescence detector (FLD, L‑2485). The separation was performed using a polar endcapped C18 reversed-phase column (4 μm Hydro‑RP 80 Å, 250×4 mm, with AQ C18 Guard Column; Phenomenex, Synergi) running a linear gradient from 100% ultrapure water, adjusted to pH 7 (± 0.05) with diluted NaOH (suprapur, Merck), to 100% methanol between 6 and 20 min. The flow increased in the same time period from 0.2 to 0.4 mL min‑1. The column oven temperature was 25 °C. Both detectors were connected in series; absorbance was recorded between 200 and 400 nm, and the fluorescence signal was measured at 260 nm excitation and 430 nm emission wavelength (ex260/em430). The excitation/emission pair used to monitor the fluorescence signal approximates the “peak A” of UV humic-like fluorescence. This peak was repeatedly found with excitation-emission-matrix spectroscopy of terrestrial organic matter and marine organic matter extracts ([Coble, 1996](#_ENREF_12); [Coble et al., 1998](#_ENREF_13)) and generally shows high fluorescence intensity. Methanol reached the detector after ~22.5 min. 10 µL of each methanol extract were injected. The average relative standard deviation of the chromatogram peak areas were 2.4 ± 1.1% as determined from eight repeated injections of a DOM extract. DAD and FLD chromatograms were blank corrected (injection of 10 µL ultrapure water).

The main benefit of the chromatographic method was to induce a physico-chemical separation (as difference in polarity) of NOM components. Investigation of the distinct fractions (DOC content, optical or molecular properties) can reveal intrinsic differences between samples that are not accessible from bulk measurements. To maximize the separation effect, a gradient from 100% water (the matrix of the original sample) to 100% methanol (the extraction solvent) was applied. The distribution of peaks within this gradient therefore reflects the full polarity spectrum of all extracted compounds. However, also size effects need to be considered in the analysis of NOM samples with reversed-phase columns ([Hutta et al., 2011](#_ENREF_34); [Lechtenfeld et al., 2011](#_ENREF_49)).

Absorbance at 210 nm was selected for the evaluation of the RP‑HPLC‑DAD spectra, according to the recently established relationship between DOC content and DOM absorption at this wavelength for individual chromatographic fractions ([Lechtenfeld et al., 2011](#_ENREF_49)). As a first approximation, the average molar extinction coefficient (ε210 nm) was calculated for the total chromatogram according to equation 1:

 ε210 nm [L mol-1 cm-1] = total peak area [L] / DOCinjected [mol] × 1 cm-1 (1)

where the total peak area refers to the integrated and blank-corrected absorbance of a sample between 4 and 36 min. DOCinjected is the amount of DOC injected on the RP column. The approximate DOC amount for each peak (DOCcalc) was then calculated from equation 2:

 DOCcalc [mol] = peak area [L] / ε210 nm × 1 cm (2)

where peak area [L] is the integrated and blank-corrected absorbance of each chromatographic peak. Absorbance at 355 nm wavelength is commonly used to characterize coastal and estuarine CDOM ([Blough and del Vecchio, 2002](#_ENREF_6)). To facilitate comparison with literature CDOM absorption coefficients (α(λ)), we estimated the absorption coefficients of the original sample, which were not measured, from equation 3:

 α(λ) [m-1] = 2.303 × ε(λ) × SPE-DOC [M] × 100 [cm m-1] / enrichment factor (3)

This approach yields only a lower limit for the CDOM absorption coefficients of the original sample because it does not consider CDOM extraction efficiencies.

FT-ICR MS measurements

DOM methanol extracts (1:1 diluted with ultrapure water) were analyzed with an FT‑ICR mass spectrometer (Apex ultra, Bruker Daltonics, Billerica, MA) equipped with a 12 T refrigerated actively shielded superconducting magnet (Bruker Biospin, Wissembourg, France). An electrospay ionization (ESI) source was used in negative ion mode (capillary voltage: +4.4 kV) with a syringe pump for continuous infusion of the sample at a rate of ~2 µL min‑1.

Fragmentation experiments via quadrupole isolation with a 1 Da isolation window and collision induced dissociation (qCID‑MS/MS) in the hexapole collision cell with Argon as collision gas (-14.5 eV) were carried out. Dissociation products were further transferred into the ICR cell and detected in a mass to charge ratio (m/z) range of 147‑2000. MS/MS mass spectra were acquired for two samples from 2009 on two high magnitude mass peaks (m/z m/z 311, C1, SML; m/z 325, E4a, SML). Although numerous peaks on a single nominal mass were detected, the high mass accuracy of FT‑ICR MS allowed the calculation and therefore identification of dissociated small molecules from mass differences in the fragmentation spectra.

Five hundred scans were added for a full spectrum and 67 to 220 for a fragmentation spectrum. FT-ICR mass spectra were externally calibrated with arginine cluster and internally recalibrated with seven masses that were repeatedly found in marine DOM samples ([Flerus et al., 2011](#_ENREF_19)). The standard deviation of the mass error of the calibration masses was below 0.03 ppm.

All peaks were singly charged ions and therefore the m/z ratio represents (molecular) mass [Da] of the compounds. Molecular formulas were calculated from the exact mass in the range of 200 – 700 Da with an accuracy ≤ ±0.5 ppm with a home-build algorithm, allowing for the following elemental compositions: C0-∞H0-∞O0-∞N0-2S0-2. For unambiguous elemental formula assignment the “nitrogen-rule” and elemental ratios O/C ≤ 1, N/C ≤ 1, H ≤ 2C+2+N ([Koch et al., 2007](#_ENREF_42); [Koch et al., 2005](#_ENREF_44)) were applied and the elemental combination N2S2 was excluded to avoid ambiguous assignments. Usually the corresponding 13C or 34S isotope mass peak magnitudes were too low for verification of the molecular formula with the isotope peak abundances (relative abundance of 13C and of 34S is 1.1% and 4.2%, respectively). Remaining ambiguously assigned mass peaks were checked according to the homologous series, i.e., chemical building block approach ([Koch et al., 2007](#_ENREF_42)). A molecular formula must be a member of a continuous “CH2” and “CH4‑O” series and the number of O‑atoms must be larger than the length of the “CH4‑O” series. To facilitate further comparison of sample pairs (SML vs ULW), we manually adjusted the lower relative peak magnitude limit (based on the highest peak of the NOM perimeter, see below) for samples E4a – 2008, ULW, C1 – 2009, SML and ULW (0.5% instead of 1%, signal to noise ratio always ≥ 4), resulting in comparable relative peak magnitude frequency distributions for all samples. This approach was necessary due to the deviating maximum peak magnitudes caused by either different total carbon content in the SPE samples or prominent ‘contaminant’ peaks (identified as O3S, O4S- and O5S-compounds, see Results section and Fig. 1). A degradation index (IDEG) was introduced by Flerus et al. ([2012](#_ENREF_20)) using relative peak magnitudes of two quintuples of peaks ubiquitous found in FT‑ICR MS samples. IDEG approximates the degradation state of solid-phase extracted DOM which is mainly dominated by heterotrophic and photochemical reworking. It is calculated according to equation 4 from the raw magnitudes of ‘POSIDEG’ (C13H18O7, C14H20O7, C15H22O7, C15H22O8, C16H24O8) and ‘NEGIDEG’ (C21H26O11, C17H20O9, C19H22O10, C20H22O10, C20H24O11) peaks, that showed a positive or negative correlation with the samples’ Δ14C values ([Flerus et al., 2012](#_ENREF_20)):

 IDEG = ∑NEGIDEG / ∑(NEGIDEG + POSIDEG) (4)

A comparison with a surfactant database (http://www.terrabase‑inc.com) revealed 71 molecular formulas in our samples that potentially represent anthropogenic surfactants. In the van Krevelen diagram, several series of homologous compounds from the database were identified that were enriched or exclusively found in the SML samples. Exclusion of method blank masses on a presence absence basis is to date the only possibility to exclude false positive molecular formulas (e.g. contaminants) from a mass list. As this study was focused mainly on naturally occurring DOM, we consequently excluded all molecular formulas from the “terrabase-inc” database from the final FT‑ICR MS dataset prior evaluation, considered to be the most conservative approach. Evidence for the presence of contaminant molecules in FT‑ICR mass spectra can be obtained with fragmentation experiments of equivocal peaks, as demonstrated in this study.

Weighted average (wa) mass and elemental ratios were calculated from the relative peak magnitudes. Using weighted averages is a common way to facilitate comparison of FT‑ICR MS spectra ([Flerus et al., 2012](#_ENREF_20); [Liu et al., 2011](#_ENREF_54); [Schmitt-Kopplin et al., 2012](#_ENREF_64)). However it is only a semi-quantitative approach assuming comparable ionization efficiencies and volatilities of the sample compounds in the ESI introduction system. The coarse shape of the spectra showed the almost Gaussian peak distribution that is characteristic for FT‑ICR mass spectra of NOM (e.g. [D'Andrilli et al., 2010b](#_ENREF_17); [Koch et al., 2005](#_ENREF_44)). The reference peak was defined as the highest magnitude peak within this perimeter, usually found between 380 and 450 Da (Fig. 1). Occasionally the base peak of the spectrum was not the maximum of the typical NOM peak distribution (i.e., the reference peak), resulting in few peaks with relative magnitudes > 100% (typically S‑compounds, Fig. 1). Double bond equivalents (DBE, representing the sum of π-bonds and rings in a neutral molecule) were calculated according to the following equation:

 DBE = 1 + ½(2C – H + N) (5)

where C, H and N is the number of carbon, hydrogen and nitrogen atoms in a molecular formula.

To evaluate molecular differences between surface microlayers from different stations (and salinities), enrichment factors (EF) for each station (SML and ULW) were calculated according to equation 6:

 EFi = [Xi]SML / [Xi]ULW (6)

where EFi is the enrichment factor for a molecular formula “i” and [Xi]SML, [Xi]ULW is the relative peak magnitude for this formula in the SML and corresponding ULW sample. Before classifying compounds as enriched or depleted, a threshold was set (enriched: EF ≥ 1.5; depleted: EF ≤ 0.67), taking into account the degree of mass peak magnitude repeatability of FT‑ICR MS measurements (< 10% peak magnitude relative standard deviation for ESI negative full scan mode; [Kido Soule et al., 2010](#_ENREF_41)). We did not assess the peak magnitude repeatability in this study, but the conservative EF thresholds require that the normalized magnitude of a compound in the SML (ULW) is at least 50% different from the ULW (SML).

Statistical analysis

For multivariate statistical analyses (Software “R”, [2012](#_ENREF_60)) we used relative peak magnitudes of all molecular formulas (after exclusion of the doubly assigned peaks). Group average cluster analysis based on the Bray-Curtis dissimilarity ([Bray and Curtis, 1957](#_ENREF_8)) and Principal Component Analysis (PCA, [Pearson, 1901](#_ENREF_59)) were carried out. Significance tests for two groups of samples were either performed with the Mann-Whitney U‑test or the Wilcox signed rank test (paired samples), based on the null hypothesis that both groups differ by less than “0” (i.e., they are equal). For *p*-values smaller than the significance level *α* = 0.05 the null hypothesis was rejected (i.e., both groups differ). Variables were always heteroskedastic and non-normally distributed, as tested with the Bartlett- and Shapiro-Wilk-test (*α* = 0.05).

Results

Physico-chemical conditions and nutrients

The water temperatures were between 20 and 22 °C in May 2009 and 25 °C in September 2008. Surface layer salinity increased from the upper estuary towards the Adriatic Sea (Table 1) because of the wedge-shaped freshwater layer in the estuary. At the Šibenik station (E4a) salinity was much higher in 2008, reflecting the very low freshwater input in late summer. The SML had consistently higher DOC concentrations compared to the ULW (mean DOC‑EF = 1.42 ± 0.29). DOC in the SML increased with salinity while macro nutrient concentrations were similar in all sample pairs (SML/ULW, relative differences < 1%) and only ULW data are reported. In 2009, nitrate and silicate concentrations at the two estuary stations E3 and E4a were 15.6 ± 1.3 µM and 36.6 ± 3.6 µM, while at the marine site C1, concentrations were 1.4 µM and 4.4 µM, respectively. Nitrite and phosphate were always below 0.5 µM and 0.1 µM, respectively. Extraction efficiencies for the SPE recovered DOC varied between 22 and 33% (Table 1), with a significantly higher amount of DOC recovered from the SML samples (average SML: 29.5 ± 3.9%; average ULW: 23.8 ± 2.0%, *p* < 0.04). The reason for the lower extraction efficiency compared to Dittmar et al. (2008) is not clear. However, the extract C/N ratios (19.4 ± 3.5) were similar to reported values for PPL ([Dittmar et al., 2008](#_ENREF_18); [Hertkorn et al., 2012](#_ENREF_32)) indicating a comparable extraction of NOM components.

HPLC

The correlation between the total absorbance of the entire chromatograms (total peak area [L]) and the DOC concentration of the extract was significant for all samples (*r* = 0.94; *n* = 8, *p* < 0.001), although a non-zero y-intercept points towards some DOC fraction not absorbing at 210 nm. The average molar extinction coefficient ε210 nm was highest for the samples from the middle station E4a and lowest for the marine station C1 (Table 2). The ε210 nm (SML) / ε210 nm (ULW) ratio as well as the calculated absorption coefficient α (*λ* = 210, 355 nm) for the original sample (i.e., considering the nominal enrichment factors of the extracts, Table 2) revealed an accumulation of CDOM in the SML samples (mean ratios α (SML) / α (ULW) = 1.9 ± 0.4 for *λ* = 210 nm and 2.4 ± 1.1 for *λ* = 355 nm). In contrast, the DOC normalized peak area of the total fluorescence (ex260/em430) showed highest values for the upper estuary station E3 and lowest values for the marine station C1, as well as generally higher fluorescence values for all ULW samples (Table 2). The decrease in total DOC normalized fluorescence is in accordance with the characteristics of the UV humic like “Peak A”, that is known to exhibit a linear negative correlation with salinity in estuaries ([Coble, 1996](#_ENREF_12)).

The DAD210 nm-chromatograms of all SML and ULW samples showed four major peaks while in some samples two additional small peaks appeared (Fig. 2). Peaks were grouped as hydrophilic (-H) and lipophilic (-L). The main features of the SML samples were an additional peak at 20.43 ± 0.17 min (4‑H, *n* = 3; not present in the 2008 sample) and a relatively higher absorbing peak at 10.73 ± 0.07 min (2‑H, *n* = 3). Independent of the sample type, all samples (except E4a – 2008) showed a major contribution of very polar, water eluting components (fractions 1‑H to 4‑H versus 5‑L and 6‑L, DAD210 nm, *p* < 0.02). Fluorescence (ex260/em430) revealed a peak at 29.04 ± 0.04 min (*n* = 3) that was only present in the ULW samples with the exception of the E4a ‑ 2008 sample.

FT-ICR MS

FT-ICR MS analyses resulted in 4,311 to 6,128 assigned molecular formulas per sample (number of total peaks per sample with S/N > 4 and 200 – 700 m/z: 11,435 – 13,702). Twenty-nine molecular formulas belonged to doubly assigned peaks with m/z > 550 Da and could not be unequivocally assigned according to the defined criteria. The summed peak magnitude of all doubly assigned peaks was only 0.01% of the total magnitude of all peaks. As a conservative approach, these peaks were removed from the final data set.

The general pattern of the molecular mass, O/C and H/C distribution of all samples (Fig. 1) resembled that of solid-phase extracted marine surface waters, as found in other studies (e.g. [Gonsior et al., 2011](#_ENREF_26); [Kujawinski et al., 2009](#_ENREF_45)). The mean elemental ratios were O/C = 0.438 ± 0.158 and H/C = 1.253 ± 0.357 (*n* = 41,953). Thirty-two percent of all formulas contained one or two nitrogen atoms, 17% contained one or two sulfur atoms and 4% contained nitrogen and sulfur (as compound classes N1S1, N2S1 or N1S2). The number of nitrogen, oxygen and sulfur peaks per sample did not show any clear trend (Table 3).

Comparing the weighted average elemental ratios of H/C and O/C (wa H/C, wa O/C) for all samples (Table 3), an increase of both ratios was found with increasing salinity and DOC concentration. The weighted average mass (wa mass) and number of peaks did not show any trend with salinity or DOC. Compared to ULW, the SML samples showed higher abundances of sulfur compounds (lower wa C/S ratio), higher saturation (higher wa H/C ratios and lower wa DBE values), and smaller wa mass. During the 2008 campaign all of these differences were particularly pronounced (Table 3).

Cluster analysis based on all molecular formulas clearly separated all samples according to their salinities (Fig. 3A). In addition, the SML samples separated from the ULW samples at the low salinity stations E3 and E4a (2009). The station E4a ‑ 2008 showed a lower similarity to the 2009 samples and also less similarity between SML and ULW. For the samples from 2009, the PCA (Fig. 3B) confirmed the clear separation between differences in salinity (PC1, 76% of variance) and between SML and ULW (PC2, 9.5% of variance).

Out of all samples, 826 ‑ 3213 molecular formulas (accounting for 5 – 48% of the summed magnitude) were enriched or appeared uniquely in the SML samples, while 655 – 2049 molecular formulas (3 – 13%) were enriched in or unique to the ULW samples (Table 4). For the 2009 samples the summed relative magnitude of enriched (depleted) compounds increased (decreased) with increasing salinity (Fig. 4) and changed consistently with the number of enriched (depleted) molecular formulas. In 2008, a pronounced enrichment of compounds in the SML sample was observed. The “unique” peaks, belonging to SML or ULW in a sample pair, were equally distributed in the van Krevelen space and had low relative magnitudes (mean for all unique peaks: 1.33 ± 0.48). Therefore the corresponding peaks in the paired sample were likely below the magnitude threshold and thus not present in the evaluation data set. Fig. 5 shows the EF values in a color coded van Krevelen diagram for station E3 and C1 distinguishing between enriched and depleted compounds.

On average, 20% of all molecular formulas in a sample and even 25 – 38% of all SML-enriched compounds contained at least one sulfur atom. The latter accounted for 28 – 44% of the summed magnitude of enriched peaks. Eight to 44% of all enriched compounds contained at least one nitrogen atom comprising 18 – 35% of the total enriched intensity. The average EF of the sulfur compound classes (“N0S1‑2”) was higher than for all molecular formulas, and it was lower for the nitrogen compound classes (“N1‑2S0”). The highest EFs for N and S-compounds were found in the 2008 samples, while the average EF of all depleted compounds was similar regardless the compound class or the sample station (Table 4).

Fragmentation experiments

To elucidate the structure of some compounds identified by FT-ICR MS, collision induced dissociation (qCID-MS/MS) experiments were performed on two high magnitude nominal masses (m/z 311, 325) from different spectra. The fragmentation pattern for m/z 311.16864 (Fig. 6, sample C1 ‑  2009, SML) strongly suggested that the oxygen was bound in a sulfonate group and not in a carboxylic group (SO2 loss, but no loss of CO2/H2O, typically observed from CID fragmentation of carboxylic acids; [Levsen et al., 2007](#_ENREF_52); [Witt et al., 2009](#_ENREF_74)). After the initial loss of C2H6 from the molecular ion [M‑H]- repeated abstractions of (CH2)n-units were detected, resulting in the base peak at m/z 183 with the molecular formula C8H8O3S (DBE = 5). A benzene ring in the molecule is a reasonable assumption (accounting for 4 DBE). Other low magnitude sulfur and non-sulfur ions were present in the full spectrum at nominal mass 311, and fragment ions of these were also detected (Fig. 6). In contrast to the fragment ions, they were also present with a higher relative magnitude in the original spectrum. The fragmentation spectrum of nominal mass 325 (E4a ‑ 2009, SML) showed fragment ions at the same mass differences but no detectable peak at m/z 170.

This approach strongly suggests that the molecular formulas of the base peaks at the nominal mass 311 and 325 (m/z 311.16864 and 325.18429) have only one major structural isomer and belong to the group of linear alkylbenzenesulfonates (LAS, which are widespread anthropogenic surfactant products). However, the exact substitution pattern and potential branching of the alkyl rest could not be resolved and might contribute to a higher degree of structural diversity for these peaks. According to these results, we assume that the other molecular formulas that belonged to the same pseudo-homologous “CH2”-series (C13+kH19+2kO3S; k = 0 ‑ 7) were true homologues and as well LAS compounds. In all samples most of the homologue molecular formulas of the LAS were also found, but with lower magnitudes. Enrichment factors for these mass peaks varied between EF = 1 and 17.

Discussion

The Krka River Estuary is a well-studied biogeochemical system (see Marine Chemistry special issue 32, 1991) with low freshwater inflow and low terrestrial organic matter load ([Cauwet, 1991](#_ENREF_10)). The karstic catchment of the Krka River is reflected in low particulate organic matter carbon to nitrogen ratios ([Svensen et al., 2007](#_ENREF_69)) suggesting mainly autochthonous production within the estuary. The persistent and strong halocline at the freshwater/saltwater interface in the microtidal estuary prevents exchange between surface brackish water and deep saline water. The halocline acts also as a barrier for autochthonous dissolved and particulate organic matter produced in the surface layer. Higher inflow of river water in May results in a shorter water residence time in the whole estuary as compared to September ([Legović, 1991](#_ENREF_50)).

Our bulk chemical data showed low DOC concentrations along the estuary transect increasing from the upper estuary to the marine station in May 2009. In the Krka Estuary non-conservative DOC mixing occurs, highlighting the importance of the autochthonous production ([Louis et al., 2009](#_ENREF_56); [Sempere and Cauwet, 1995](#_ENREF_65)). The very low phosphate concentrations throughout the whole estuary and lipid and fatty acid analysis (B. Gašparović et al. unpubl.) indicate growth limitation and point towards low production and post spring-bloom conditions. Compared to the upper estuary (station E3), nitrate was slightly elevated at the middle station E4a, which is closest to the only larger city along the estuary. Human activity may induce higher nutrient levels ([Legović et al., 1994](#_ENREF_51)), although this was not observed in our phosphate data.

Chromatographic and optical properties

DOC and total absorbance (*λ* = 210 nm) were always higher in the microlayer than in the underlying water. The absorption coefficients *α*(355 nm) fell in the range of previously reported values for Adriatic Sea CDOM ([Berto et al., 2010](#_ENREF_4)) and revealed high EFs for CDOM in the SML ranging from 4.0 (station E3) to 1.8 (station C1) which is clearly higher than the enrichment of total DOC (mean EF of 1.42). Such high CDOM-EFs agree with data from slick samples ([Blough, 1997](#_ENREF_5); [Wurl et al., 2009](#_ENREF_76)), although visible surface slicks were not present at the sampling time. Considering enhanced photochemical degradation of CDOM in the SML a continuous CDOM enrichment has to occur in the SML ([Wurl et al., 2009](#_ENREF_76)).

The first two peaks in the DAD210 nm chromatograms (1-H, 2-H) showed the highest differences between SML and ULW samples. In addition to a pure polarity separation, the first two peaks partly reflect a size exclusion separation with a larger average molecular size for peak 1-H compared to peak 2-H ([Hutta et al., 2011](#_ENREF_34); [Lechtenfeld et al., 2011](#_ENREF_49)). This is a result of the narrow average pore size diameter of the RP column with only 80 Å, resulting in an exclusion of the largest molecules or strongly bound aggregates. The peaks 1-H and 2-H therefore elute prior the dead volume of sample methanol and their separation resulted from a mixed mode separation mechanism preventing a direct correlation between hydrophobicity/molecular size and retention time. However, in all SML samples the proportion of the calculated DOC content (%‑DOCcalc) is higher in peak 2-H than in peak 1-H likely resulting from a higher contribution of lower molecular weight compounds in the SML (Fig. 7). This is also supported by a smaller weighted average mass of SML compared to ULW compounds as detected by FT‑ICR MS.

The trend of smaller average molecular size in the SML sample was especially evident from the 2008 samples from the middle station E4a. There, in the ULW sample, peaks 1-H and 2-H were shifted towards lower retention time (i.e., higher average size compared to the 2009 ULW samples). The E4a ‑ 2009, SML sample showed in contrast a higher DOCcalc contribution and pronounced shift of peak 2-H to lower molecular size (compared to the 2008 ULW and 2009 SML samples). These results likely reflect an enhanced photochemical degradation of organic matter in the SML, presumably combined with enhanced microbial breakdown ([Obernosterer et al., 2005](#_ENREF_58)). Hence, the pronounced accumulation of small breakdown products in the SML in September 2008 can be explained with the extended exposure to such degradation processes due to the longer water residence time in summer ([Legović, 1991](#_ENREF_50)). This is also supported by size-exclusion chromatography, which showed a unique peak of low molecular size compounds only appearing in the SML sample from 2008 (data not shown).

The total DOC normalized fluorescence decreased greatly from the freshwater E3 to the marine C1 station (on average 73% reduction for SML and ULW samples, Table 2). We assume that this change was due to differences in autochthonous production in the estuary ([Ahel et al., 1996](#_ENREF_1); [Cetinic et al., 2006](#_ENREF_11); [Svensen et al., 2007](#_ENREF_69)) and that the degradation of fluorophores during the transport from station E3 to C1 is responsible for the decrease in fluorescence. We further assume that the degradation of only small amounts of terrestrial humic-like material ([Blough and del Vecchio, 2002](#_ENREF_6)) from the karstic watershed of the Krka river is a minor contribution to the observed fluorescence decrease, in contrast to humic-rich river estuaries ([Yamashita et al., 2008](#_ENREF_81)). Moreover, the DOC normalized total fluorescence ratio SML/ULW was always below one (Table 2). This indicates that fluorophores with either different quantum yield or higher abundance were present in the ULW samples, strong photobleaching in the SML took place or quenching effects due to the different compositions of both phases were dominant.

The occurrence of the unique fluorescence peak in the ULW samples at 29 min (not detected in the 2008 samples) presumably reflects fluorophores derived from primary production ([Koch et al., 2008](#_ENREF_43)). The contribution of this peak to the total fluorescence was highest in the freshwater E3 sample and lowest in the marine C1 sample. This might be a discrimination effect in the microlayer enrichment mechanism (due to the changing ionic strength) or a consequence of degradation along the estuarine transport. However, this peak was also found in deeper water layers of the estuary (data not shown), suggesting that this fraction is also rapidly degraded in the SML.

Molecular characterization

FT-ICR MS analyses revealed that each SML sample had higher wa H/C (from +0.01 to +0.09) and lower wa mass (from -5.7 to -14.9 Da) compared to the ULW samples (Table 3). However, based on the complete sample set, there was no significant trend between these molecular parameters and salinity or DOC. Only the classification of all molecular formulas in each SML/ULW sample pair into “enriched/depleted in the SML” can explain these results in terms of microlayer chemical characteristics (Fig. 5). Applying this approach, changes in numbers and proportional intensity of enriched/depleted compounds with increasing salinity were revealed in the estuary (Fig. 4): At low salinities (i.e., low ionic strength) only compounds with very high H/C ratios were enriched in the SML. In contrast, at the high salinity marine station C1, the mean H/C ratio of compounds enriched in the SML and those enriched in the ULW were more similar (Table 5). The change in the H/C ratios of the enriched and depleted compounds points towards a separation mechanism of hydrophobic constituents at the phase transition between ULW and SML.

The concept of “salting-out” of hydrophobic substances ([Setschenov, 1889](#_ENREF_66)) implies that the water solubility of hydrophobic molecules decreases with increasing ionic strength ([Xie et al., 1997](#_ENREF_80)). Applied to the ULW/SML system, we assume that only at high ionic strength, the water-solubility of “moderately” hydrophobic (amphiphilic) substances is sufficiently reduced to accumulate in the SML. The salt-mediated, additional enrichment of compounds with intermediate H/C values therefore reduces the average H/C ratio of all enriched compounds at the marine station. Indications that the FT‑ICR MS molecular H/C ratio can be interpreted as a measure of hydrophobicity of compounds (at constant O/C ratio) were derived from coupled RP‑HPLC‑FT‑ICR MS experiments ([Koch et al., 2008](#_ENREF_43); [Liu et al., 2011](#_ENREF_54)).

Consistent with the results from the HPLC analyses and the FT‑ICR MS data from the total samples, the mean molecular mass of all SML enriched compounds was lower than for the total of all peaks and especially lower than for the ULW enriched compounds (i.e., depleted in the SML) in the corresponding paired sample (Table 5). This agrees with previous reports of photochemically produced and enriched low-molecular-weight compounds in surface microlayers ([Schmitt-Kopplin et al., 2012](#_ENREF_64); [Zhou and Mopper, 1997](#_ENREF_83)).

In addition, the contribution of S-compounds (on number and intensity basis) to all SML enriched molecular formulas was higher in the low salinity samples than in the high salinity samples, while the proportion of N‑compounds increased slightly with salinity (Fig. 8). Moreover, the difference in the H/C ratio and mass between the enriched and depleted N‑compounds is always larger than the corresponding difference between the S‑compounds. This suggests that, compared to nitrogen compounds, the size and polarity distribution of sulfur bearing compounds is more similar in SML and ULW. Thus, S‑compounds are less influenced by the “salting out” effect along the estuary.

The variation between enriched and depleted compounds in the H/C dimension (the effect was less pronounced for the O/C ratios) was also demonstrated by the multivariate statistical analysis. The difference in the H/C ratios (Table 5) and the distance between SML and ULW samples on the PC2 axis (Fig. 3B) reflect the difference in hydrophobicity. It should be noted that there is only little compositional overlap of the enriched/depleted substances between the four sample pairs, which can be attributed partly to the defined relative magnitude and EF thresholds.

Microlayer enriched compounds with a similar range of H/C and O/C ratios and low molecular mass were also found in the SML off the coast of Mallorca Island ([Morales-Cid et al., 2009](#_ENREF_57)) and a study on the sea-air phase transfer of organic matter ([Schmitt-Kopplin et al., 2012](#_ENREF_64)). Air bubbles mediate an enhanced transport of surface-active compounds from the bulk phase to the SML and further into the atmosphere. Active enrichment due to breaking waves is also the reason why at rough wind conditions, mean enrichment factors of surfactants can be even higher than at calm winds ([Wurl et al., 2011](#_ENREF_78)). However, this did not influence the sample composition in our study, due to prevailing calm wind conditions during sampling.

The late summer 2008 sample from the middle station E4a could be described as a superposition of the high salinity sample C1 and the anthropogenically influenced middle estuary station from 2009. The different environmental conditions (longer water residence time, higher solar radiation dose, higher temperature) were likely reflected in the higher wa H/C, lower wa O/C ratios, wa mass and wa DBE values as well as high DOC concentration for both 2008 ‑ E4a samples. Also, a set of high magnitude (> 5% relative peak magnitude), highly enriched (EF > 2.5) compounds was found (Fig. 1), corresponding to saturated (CnH2nCOOH) and mono unsaturated (CnH2n-2COOH) fatty acids, not present in the 2009 samples. If we consider that enrichment in the SML is dependent on the polarity of individual molecules (reflected by the H/C ratio) and the ionic strength of the medium, these results are in accordance with the proposed physico-chemical separation at the phase boundary. Frka et al. ([2009](#_ENREF_22)) reported surface active substance concentrations at the Šibenik site that were three times higher in the SML and five times higher in the ULW in summer than in winter. These substances accumulate in the estuarine SML during the low run-off summer months, being more hydrophobic than in winter. Reports on the enrichment of hydrophobic lipid-like compounds or fatty acids (having high H/C and low O/C ratios) in SML samples are ambiguous (e.g. [Gašparović et al., 2007](#_ENREF_25); [Lass and Friedrichs, 2011](#_ENREF_48)). However, based on mass spectrometric analysis, Frew et al. ([2006](#_ENREF_21)) reported an enrichment of surface-active lipids in slicked SML samples.

Seasonal differences were also reflected in very high EFs of some compounds in the September 2008 sample set compared to the May 2009 samples from the same station. The E4a – 2008 SML sample showed a clearly higher abundance of unique high magnitude, high H/C, and low O/C compounds which cannot be explained alone with the salinity-trend for the enrichment of substances. A set of high magnitude, sulfur bearing compounds contributed mainly to this enrichment.

Our fragmentation experiments revealed the occurrence of LAS in the SML, most pronounced in the 2008 sample close to the city of Šibenik, which is in agreement with earlier studies ([Ahel and Terzic, 2003](#_ENREF_2)). Calculation of concentrations of these substances was not possible, as FT‑ICR MS data do not allow quantitative conclusions but the magnitude distribution of the LAS peaks in our samples (Fig. 9) correspond well to the distribution determined by the industrial production process ([Alzaga et al., 2003](#_ENREF_3)). A comparison of the relative peak magnitudes for each sample pair revealed a high enrichment in the SML for some LAS homologues for both high saline samples (E4a – 2008 and C1 – 2009; Fig. 9). Relative magnitudes and enrichment were most pronounced in the 2008 sample, probably as a result of the longer water residence time in the estuary ([Legović, 1991](#_ENREF_50)). This strongly suggests enhanced enrichment of LAS in the microlayer with increasing salinity aided by the “salting-out” effect and suppression of the heterotrophic metabolic activity in higher saline waters ([Terzic et al., 1992](#_ENREF_70); [Alzaga et al.,](#_ENREF_3) 2003).

Other highly SML enriched sulfur compounds in the 2008 samples may also be passively or actively enriched from lower layers or may originate from autochthonous production in the surface layer. However, primary production was unusually low during September (B. Gašparović et al. unpubl.). The contemporary view on the molecular structuring of SML ([Cunliffe et al., 2011](#_ENREF_15)) requires a hydrated layer of a heterogeneous polymeric network. Carbohydrates ([Kuznetsova et al., 2005](#_ENREF_46); [Williams et al., 1986](#_ENREF_73)) and TEP ([Cunliffe et al., 2009](#_ENREF_14); [Wurl et al., 2009](#_ENREF_76)) are enriched in SML with a high fraction of sulfate ester groups ([Wurl and Holmes, 2008](#_ENREF_75)). However, high molecular weight, oxygen-rich compounds were not detected in our FT‑ICR MS samples. Our findings based on FT‑ICR MS fragmentation and database comparison hence necessitate very careful interpretation of sulfur containing molecular formulas as well as other potential non-ionic surfactant masses in future FT‑ICR MS studies of solid-phase extracted NOM.

An approach to assess the general degradation state of an NOM sample is the degradation index (IDEG, Eq. 4; [Flerus et al., 2012](#_ENREF_20)). In our study the SML samples had generally higher IDEG-values than the ULW samples, indicating a higher degree of degradation in the SML. Moreover, the 2008 samples had higher values than the 2009 samples. An explanation for the higher degree of degradation could be the enhanced photochemical and microbial reworking in the SML and the decoupling between autotrophic production and transformation of organic matter in this particular physico-chemical environment (as supported by the 29 min FLD peak, [Obernosterer et al., 2005](#_ENREF_58); [Santos et al., 2011](#_ENREF_62)). Therefore, the IDEG parameter might also be well applicable to characterize the degradation state of sea surface microlayers. Our ongoing research aims at understanding the molecular mechanisms and biogeochemical causes for the observed differences in the IDEG parameter.

Major processes that determine the molecular composition and hence the degradation state of NOM in the surface microlayer with respect to the bulk water phase are depicted in Fig. 10: The SML is characterized by a strong enrichment of bacterial biomass ([Sieburth et al., 1976](#_ENREF_67)) and predominating heterotrophic processes ([Reinthaler et al., 2008](#_ENREF_61); [Santos et al., 2011](#_ENREF_62)). Together with the photoinhibition of photoautotrophs in the SML (due to high UV‑B radiation) a decoupling of production and degradation can occur ([Obernosterer et al., 2005](#_ENREF_58)). The pronounced exposure to sunlight in stratified estuaries and low wind conditions then favors the photochemical breakdown of DOM molecules in the SML ([Tilstone et al., 2010](#_ENREF_71)). Physico-chemical processes further determine the composition of the SML and ULW, such as diffusion, bubble entrainment ([Wurl et al., 2011](#_ENREF_78)), “salting-out” of hydrophobic molecules ([Xie et al., 1997](#_ENREF_80)) and spontaneous vertical phase separation and horizontal segregation of surface active substances ([Frka et al., 2012](#_ENREF_23)). However, sorption of organic compounds on suspended particles also changes the partitioning between water, solid phase and the hydrophobic surface microlayer ([Brunk et al., 1997](#_ENREF_9); [Gschwend and Schwarzenbach, 1992](#_ENREF_27)) and might lead to further molecular fractionation between these phases in shelf waters ([Boehm, 1980](#_ENREF_7)). The chemical observations presented in this study are a superposition of all these effects, determining the equilibrium state of hydrophobic and hydrophilic substances between SML and ULW. Our current research aims at resolving the relative contribution of each effect on the molecular level distribution of compounds.

Conclusions

Our ultra-high resolution mass spectrometry results on solid-phase extracted DOM from surface microlayers and underlying waters in the karstic Krka river estuary demonstrated that the SML is a layer with considerable compositional analogy to the ULW. However, specific differences in the molecular composition were attributable to the salinity gradient, a factor which is so far rarely considered in SML studies. In addition to the highly dynamic chemical and physical character of the SML, surface microlayers sampled in estuarine or coastal zones are particularly prone to anthropogenic influences. Hydrophobic and surface-active compounds released from ship traffic and wastewater discharge potentially influence the distribution of substances at the sea surface, which are not covered by the analytical window of conventional methods for surface microlayer studies. The presented study combines the benefits of high resolution molecular analysis of DOM with the potential to identify sampling site specific anthropogenic contributions. For a comprehensive understanding of the processes in the SML and their global significance for e.g. the accumulation and degradation of pollutants and the sea-air exchange of energy and matter, all of these factors need to be considered.

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 Figure captions

Fig. 1. A: Negative ESI FT-ICR mass spectra of a bulk water (ULW) and a microlayer sample (SML). SPE samples are from the middle station in the Krka estuary (E4a – 2008). B: Visual representation of all assigned molecular formulas for the ULW (*n* = 4311) and the SML (*n* = 4769) sample in a van Krevelen plot. Molecular hydrogen to carbon (H/C) vs. oxygen to carbon (O/C) ratios are plotted according to the relative peak magnitude. Prominent sulfur (“×”) and CnH2nCOOH/CnH2n-2COOH (“o”) peaks are indicated in the ULW sample and marked in the van Krevelen plots (black arrows and circles). Plots were prepared using Ocean Data View (R. Schlitzer, http://odv.awi.de).

Fig. 2. Reversed phase high performance liquid chromatograms of microlayer and underlying water extracts (E3, SML and E3, ULW) with diode array (200‑350 nm, left Y-axis) and relative fluorescence (FLD, ex260/em430 nm, black line, right Y-axis) signals. Diode array intensities were converted to molar extinction coefficient εOC and displayed as color scale for both samples. For peak labels, see text.

Fig. 3. Multivariate statistical analysis of untransformed FT-ICR MS peak magnitudes of all molecular formulas (*n* = 41953). A: cluster analysis (Bray‑Curtis similarity) for all samples. B: principal component analysis (PCA) for the 2009 samples (SML = microlayer, solid lines; ULW = underlying water, dashed lines). The circle size specifies the salinity of each sample.

Fig. 4. Magnitude proportions of all molecular formulas that were enriched (EF > 1.5) or uniquely found in the microlayer (SML) or underlying water (ULW) sample of each station (ULW enriched corresponds to EF < 0.67) with the corresponding salinities.

Fig. 5. Van Krevelen visualization of FT‑ICR MS derived molecular formulas that were enriched or depleted in the surface microlayer of a low salinity sample (E3, S = 6) and a high salinity sample (C1, S = 35), not including unique peaks for clarity. The enrichment factor for each peak is color coded (same scale for all panels). In the top left panel, the location of anthropogenic surfactant homologues from a database (http://www.terrabase‑inc.com) are displayed as neutral molecules in the van Krevelen space (APEO = alkylphenol ethoxylates, (L)AS = (linear) alkylbenzenesulfonates). Plots were prepared using Ocean Data View (R. Schlitzer, http://odv.awi.de).

Fig. 6. Collision induced dissociation (qCID-MS/MS) spectrum of nominal mass 311 of a microlayer sample (C1 ‑ 2009, SML) with exact m/z values for fragment ions. Fragments from calculated mass differences are displayed together with possible neutral structures of the major ions: undecylbenzenesulfonic acid (A, molecular ion peak), non‑8‑enylbenzenesulfonic acid (B), undecylphenol (C) and styrenesulfonic acid (D, base peak). “×” = major additional sulfur compounds (O4S, O5S), which possibly resulted from fragmentation of additional molecular ions at the isolation nominal mass. Other peaks present in the original spectrum C1 – 2009, SML at mass 311 are shown in the insert with assigned molecular formulas.

Fig. 7. Relative proportions of the calculated DOC amount (DOCcalc) for the chromatographic peaks for all samples with the corresponding total DOC concentration in the extract (SPE-DOC). For peak names refer to Fig. 2. Peaks that do not appear in all samples are highlighted (4-H and 6-L, black boxes).

Fig. 8. Enrichment properties of different compound classes (CHO, CHNO, CHOS) for all stations. A: relative magnitude contribution to all enriched and unique compounds in the SML and ULW together with the salinity of the SML sample. B: mean molecular H/C ratio and C: mean molecular mass for the SML or ULW enriched compound classes. CHNOS as well as ULW enriched compounds for E4a – 2009 were omitted for this figure due to their low abundances. Mean ratios were used to highlight the differences in the molecular composition, as the peaks were selected by their EF and not their relative magnitude.

Fig. 9. Relative peak magnitude distributions in the surface microlayer and enrichment factors for the proposed linear alkylbenzenesulfonate (LAS) homologues xC7 – xC14 in the Krka Esturay. The “x” indicates that the position of the benzene sulfonate group is unknown and the length of the alkyl rest is expressed as “C” and number. No enrichment factors were calculated for xC7 – xC9 and xC14 as the magnitudes for these homologues in the ULW samples were below the threshold.

Fig. 10. Summarizing sketch of the dominating processes in the SML and ULW and their consequences for the observed molecular characteristics of the DOM samples. Note that the “boundary” between ULW and SML is a transition layer (Zhang et al. 2003) but the molecular information is obtained from discrete samples.

Fig. 1



Fig. 2



Fig. 3



Fig. 4



Fig. 5



Fig. 6



Fig. 7



Fig. 8



Fig. 9



Table 1. Overview and general data of all samples. SML: surface microlayer, ULW: underlying water, IDEG: molecular degradation index after Flerus et al. (2012).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Sample | Location | Sample-type | Temperature[°C] | Salinity | DOC[µM] | SPE efficiency [%] | IDEG |
| E4a ‑ 2008 | middle estuary | SML | 25 | 30 | 181.3 | 26.8 | 0.36 |
| ULW | 25 | 30 | 101.2 | 22.4 | 0.32 |
| E3 ‑ 2009 | upper estuary | SML | 21.3 | 1.9 | 89.3 | 33.6 | 0.21 |
| ULW | 21.3 | 1.8 | 59.8 | 23.3 | 0.20 |
| E4a ‑ 2009 | middle estuary | SML | 22 | 6.3 | 97.1 | 31.9 | 0.23 |
| ULW | 22 | 6.3 | 84.2 | 26.8 | 0.20 |
| C1 ‑ 2009 | marine station | SML | 20.3 | 34.9 | 174.5 | 25.6 | 0.21 |
| ULW | 20.3 | 34.8 | 142.1 | 22.9 | 0.17 |

Table 2. Extract average molar extinction coefficient ε210 nm and calculated absorption coefficients α(λ). Total DOC normalized fluorescence (FLU) is relative to the sample with the most intense signal (100%) and normalized to the injected DOC amount.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Sample | Sample-type | ε210 nm[L mol-1 cm-1] |  α(λ) in original sample [m-1]  | Ratio α(λ) SML / ULW | FLU[%] | Ratio FLUSML / ULW |
| 210 nm | 355 nm | 210 nm | 355 nm |
| E4a – 2008 | SML | 527.6 | 5.90 | 0.27 | 2.1 | 1.6 | 58.0 | 0.76 |
| ULW | 548.4 | 2.86 | 0.18 | 73.2 |
| E3 – 2009 | SML | 488.7 | 3.52 | 0.27 | 2.3 | 4.0 | 78.4 | 0.78 |
| ULW | 441.6 | 1.50 | 0.07 | 100.0 |
| E4a – 2009 | SML | 531.4 | 3.93 | 0.18 | 1.5 | 2.2 | 70.0 | 0.80 |
| ULW | 456.0 | 2.48 | 0.08 | 87.9 |
| C1 – 2009 | SML | 424.2 | 4.47 | 0.13 | 1.7 | 1.8 | 23.0 | 0.94 |
| ULW | 343.8 | 2.64 | 0.07 | 24.5 |

Table 3. FT-ICR MS weighted average (wa) molecular parameters of the complete SPE sample set. H/C: hydrogen to carbon ratio, O/C: oxygen to carbon ratio, DBE: double bond equivalents. Number-percentage of CHO, CHNO, CHOS and CHNOS compounds to all detected ions.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Sample | Sample-type | Assigned formulas | wa H/C | wa O/C | wa mass [Da] | wa DBE | CHO peaks [%] | CHNO peaks [%] | CHOS peaks [%] | CHNOS peaks [%] |
| E4a – 2008 | SML | 4769 | 1.440 | 0.364 | 415.71 | 7.03 | 49.5 | 27.1 | 19.9 | 3.4 |
| ULW | 4311 | 1.348 | 0.400 | 421.52 | 7.98 | 52.3 | 31.4 | 14.0 | 2.1 |
| E3 –2009 | SML | 5420 | 1.231 | 0.461 | 452.32 | 9.45 | 47.5 | 33.1 | 16.4 | 2.9 |
| ULW | 6128 | 1.217 | 0.450 | 459.13 | 9.82 | 48.4 | 34.3 | 14.1 | 3.2 |
| E4a –2009 | SML | 5247 | 1.251 | 0.467 | 448.25 | 9.11 | 47.0 | 32.0 | 18.3 | 2.7 |
| ULW | 5558 | 1.229 | 0.471 | 463.17 | 9.60 | 47.7 | 32.5 | 16.3 | 3.5 |
| C1 –2009 | SML | 5530 | 1.266 | 0.484 | 457.11 | 9.00 | 42.5 | 33.7 | 19.4 | 4.5 |
| ULW | 4972 | 1.253 | 0.492 | 466.24 | 9.26 | 44.1 | 33.1 | 17.4 | 5.4 |

Table 4. Average enrichment factors (EF) for all, CHNO and CHOS compounds for the enriched (enr.) and depleted (depl.) compounds. Values in brackets are the number of respective peaks, not including the unique peaks. For E4a – 2008 depl., values were omitted (n.a.; only one peak below EF = 0.67). Unique peaks include all compound classes.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| EF forsample |    all compounds    |  CHNO compounds  |  CHOS compounds  |   Unique peaks (all)   |
| enr. | depl. | enr. | depl. | enr. | depl. | enr. | depl. |
| E4a ‑ 2008 | 3.36 | n.a. | 2.14 | n.a. | 5.97 | n.a. |  |  |
| (1872) | n.a. | (386) | n.a. | (303) | n.a. | (1341) | (882) |
| E3 ‑ 2009 | 2.16 | 0.58 | 1.72 | 0.61 | 2.57 | 0.57 |  |  |
| (107) | (622) | (32) | (97) | (35) | (43) | (719) | (1427) |
| E4a ‑ 2009 | 1.94 | 0.60 | 1.69 | 0.60 | 2.33 | 0.61 |  |  |
| (204) | (497) | (66) | (75) | (55) | (95) | (679) | (990) |
| C1 ‑ 2009 | 1.97 | 0.58 | 1.78 | 0.54 | 2.73 | 0.60 |  |  |
| (591) | (44) | (245) | (11) | (91) | (18) | (1173) | (615) |

Table 5. Mean values and standard deviation for H/C, O/C, mass and DBE for all compounds that were enriched (enr.) or depleted (depl.) in the SML, not including the unique peaks. For E4a – 2008 depl., values were omitted (n.a.; only one peak below EF = 0.67).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sample |         H/C         |         O/C         |      mass [Da]      |         DBE         |
| enr. | depl. | enr. | depl. | enr. | depl. | enr. | depl. |
| E4a ‑ 2008 | 1.454 | n.a. | 0.371 | n.a. | 405.3 | n.a. | 6.8 | n.a. |
| ±0.295 | n.a. | ±0.152 | n.a. | ±103.4 | n.a. | ±3.5 | n.a. |
| E3 ‑ 2009 | 1.476 | 1.117 | 0.487 | 0.382 | 401.6 | 483.9 | 6.3 | 11.9 |
| ±0.363 | ±0.299 | ±0.186 | ±0.143 | ±86.1 | ±131.6 | ±5.0 | ±3.4 |
| E4a ‑ 2009 | 1.462 | 1.113 | 0.521 | 0.497 | 386.3 | 566.0 | 5.9 | 12.9 |
| ±0.311 | ±0.266 | ±0.186 | ±0.127 | ±81.7 | ±97.7 | ±3.5 | ±4.1 |
| C1 ‑ 2009 | 1.383 | 1.347 | 0.5 | 0.464 | 403.7 | 480.2 | 6.9 | 9.7 |
| ±0.276 | ±0.425 | ±0.167 | ±0.165 | ±88.9 | ±155.3 | ±3.1 | ±8.1 |