Journal of Chromatography A, 1409 (2015) 218–225

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Combining asymmetrical flow field-flow fractionation with on- and off-line fluorescence detection to examine biodegradation of riverine dissolved and particulate organic matter

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ARTICLE INFO

Article history:
Received 23 May 2015
Received in revised form 15 July 2015
Accepted 18 July 2015
Available online 21 July 2015

Keywords:
Dissolved organic matter (DOM)
Microbial degradation
Asymmetrical flow field-flow fractionation
AF4
EEM

ABSTRACT

This study demonstrated that asymmetrical flow field-flow fractionation (AF4) coupled with on-line UV and fluorescence detection (FLD) and off-line excitation-emission matrix (EEM) fluorescence spectroscopy can be employed to analyze the influence of microbial metabolic activity on the consumption and production of freshwater organic matter. With the AF4 system, organic matter is on-line enriched during a focusing/relaxation period, which is an essential process prior to separation. Size-fractionated chromophoric and fluorophoric organic materials were simultaneously monitored during the 30-min AF4 separation process. Two fractions of different sizes (dissolved organic matter (DOM) and particulate organic matter (POM)) of freshwater samples from three locations (up-, mid-, and downstream) along the Han River basin of Korea were incubated with the same inoculum for 14 days to analyze fraction-specific alterations in optical properties using AF4-UV-FLD. A comparison of AF4 fractograms obtained from pre- and post-incubation samples revealed that POM-derived DOM were more susceptible to microbial metabolic activity than was DOM. Preferential microbial consumption of protein-like DOM components concurred with enhanced peaks of chromophoric and humic-like fluorescent components, presumably formed as by-products of microbial processing. AF4-UV-FLD combined with off-line identification of microbially processed components using EEM fluorescence spectroscopy provides a powerful tool to study the relationship between microbial activity and composition as well as biodegradability of DOM and POM-derived DOM from different origins, especially for the analysis of chromophoric and fluorophoric organic matter that are consumed and produced by microbial metabolic activity. The proposed AF4 system can be applied to organic matter in freshwater samples having low concentration range (0.3–2.5 ppm of total organic carbon) without a pre-concentration procedure.

1. Introduction

Natural organic matter (NOM) consists of compounds formed through the degradation of several source materials, such as plants, animals, and other organisms, or it can originate from land use or other types of anthropogenic activities. Depending on origin, NOM has different compositions, sizes, and optical characteristics [1,2]. Due to precipitation and other natural processes, this organic matter flows into the riverine ecosystem in a suspended state and is transported by the stream of surface water. Organic matter in rivers has a broad size distribution that can be divided into two groups: dissolved organic matter (DOM) that passes through a filter with a nominal pore size ranging from 0.2 to 0.7 μm and particulate organic matter (POM) that is collected as the retentate on the filter. In a more concrete definition, DOM is composed of nano- to micrometer-sized colloidal particles suspended in a water stream, and POM-derived DOM (or P-DOM hereafter) is the organic matter attached to the suspended sediment. These organic materials play various important roles in biogeochemical processes in riverine and marine ecosystems, such as metal chelation, transportation of pollutants, governance of aquatic photochemistry, and as nutrition and energy sources for biomes [3–7]. Riverine DOM and POM represent a critical link in the global carbon cycle, respiring large amounts of organic carbon exported from terrestrial sources during transport to the oceans [8,9].

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http://dx.doi.org/10.1016/j.chroma.2015.07.074
0021-9673/

Organic matter is classified into different types such as hydrophobicity, size, optical properties, and biodegradability [10]. Among them, aquatic organic matter is categorized by two types based on biodegradability: biodegradable organic matter (BOM), which can be degraded by microorganisms, and refractory humic substances, which are resistant to biodegradation [11–13]. Small fractions of both DOM and POM are digested by microorganisms and called biodegradable DOM and POM, respectively (BDOM & BPOM). Therefore, investigation of the biodegradability of organic matter is important in understanding the role of organic matter in the riverine ecosystem and the global carbon cycle. A number of studies have been conducted to elucidate the process of biodegradation in relation to source dependent degradation by microorganisms and accompanied changes in organic matter in terms of size, chemical composition, and optical properties [14–21]. Various analytical techniques such as capillary electrophoresis (CE), liquid chromatography, field-flow fractionation (FFF), mass spectrometry (MS), and fluorescence spectroscopy have been utilized to characterize organic matter from soil, riverine, and marine environments [22–25]. Among these techniques, fluorescence spectroscopy has been powerfully utilized to obtain important molecular information on organic matter. In particular, excitation–emission matrix (EEM) fluorescence spectroscopy has proven to be a useful tool to distinguish different types of organic matter based on fluorescence wavelengths [26] and has been widely utilized in practical applications such as evaluation of wastewater treatment [27,28]. For the determination of biodegradability of organic matter, two typical methods are utilized. Parallel factor analysis (PARAFAC) characterizes different aquatic organic matter using the statistical fluorescence profiling. The other method measures the amounts of carbon and nitrogen generated in the course of microbial respiration [12–19]. However, information about the changes in organic matter is not readily obtained from these methods. PARAFAC of organic matter has some pitfalls as it only shows the overall statistical profile according to the type of organic matter such as protein-like or humic-like, and it does not provide information on the size-dependent changes in organic matter or on the creation of different types of organic matter.

Flow field-flow fractionation (FlFFF) is an elution-based separation technique that takes place in a thin empty channel space through application of two perpendicular flow streams (one for migration of sample components and the other for cross-flow to retard sample migration) [29,30]. FlFFF takes advantage of the diffusion characteristics of particles or macromolecules in which particles with faster diffusion (or smaller Stokes' diameter) are distributed against the channel wall with a center of gravity (cg) higher than that of slower diffusion (larger particles); therefore, smaller particles elute earlier than larger particles in a flow stream with parabolic flow velocity profiles. Among variants of FlFFF, asymmetrical flow field-flow fractionation (AF4) has been widely utilized for size separation and characterization of proteins, cells, viruses, and water-soluble polymers [31–34]. In particular, FlFFF was employed to study the molecular weight distribution of natural organic matter [35,36], the relationship between elemental composition and size of natural colloids in the environment [37], and variations in fluorescence of chromophoric DOMs using off-line EEM fluorescence spectroscopy [38]. However, studies on the biodegradation of organic matter (BOM) using FlFFF are rare.

This study demonstrated that AF4 combined with on- and off-line FL detection can be utilized as a powerful tool to characterize chromophoric and fluorophoric DOM components of various size and origin from freshwater samples. The AF4 system employed in this study allows such a powerful detection owing to the on-line enrichment of low concentration sample before analysis and having an option to select the type of organic matter to analyze. Two different sized fractions (DOM and POM) of freshwater samples from three locations (up-, mid-, and downstream) along the Han River basin of Korea were incubated with the same inoculum for 14 days in order to analyze fraction-specific alterations in optical properties using AF4-UV-FLD and EEM fluorescence spectroscopy. Organic matter in water samples was on-line enriched in the AF4 channel prior to separation, and direct observation of the size and population of organic matter present in freshwater were readily achieved in order to examine microbially induced alterations in optical properties of DOM and POM-derived DOM (P-DOM). The present study focused on two specific types of fluorescent organic matter components, protein-like and humic-like, which were selectively monitored at each unique fluorescence wavelength by examining modifications in the size of organic matter from up-, mid-, and downstream river water induced by biodegradation.

2. Experimental

2.1. Sample preparation

Stream and river water samples were collected from three different locations in the Han River basin of Korea: a forested headwater stream in the Haean Basin, Yanggu (38°15′ N, 128°7′ E; hereafter called "upstream"); the Mandae stream, an agricultural stream draining the entire Haean Basin (38°16′ N, 128°9′ E; "midstream") on August 18; and a downstream reach of the Han River near a small island called Bamseom (37°32′ N, 126° 55′ E; "downstream") on August 19, 2014. Samples were collected following a small monsoon rainfall event with a total precipitation of 14 mm such that hydroclimatic conditions around the sampling dates represented weather conditions typical of the summer monsoon period. Water at a depth of 10–30 cm from the stream surface was collected in acid-cleaned amber glass bottles using a Masterflex® E/S™ portable field pump from Cole-Parmer (Vernon Hills, IL, USA), equipped with acid-cleaned silicone tubing. Samples were stored in the dark on ice until being filtered in the laboratory. Measured values of total organic carbon (TOC) of each sample are listed in Table S1 of Supplementary data.

Sample preparations are shown in the schematic chart in Fig. 1. The DOM and POM fractions were prepared by filtering water samples using a 0.2-μm Whatman® Nuclepore Polycarbonate membrane and a 0.7-μm Whatman® glass microfiber filter (grade GF/F), respectively, both from GE Healthcare Life Sciences (Piscataway, NJ, USA). Since POM contains organic molecules attached to suspended sediment, retrieval of P-DOM was accomplished by lyophilizing the filter paper with the retentate, cutting it into small pieces, and immersing them in ultrapure water to detach particles from the filter paper. A standard fulvic acid sample was prepared by dissolving Suwannee river fulvic acid standard from IHSS (International Humic Substance Society) in ultrapure water to a ~1 mg/L solution, which was then filtered through a 0.2-μm polycarbonate membrane. Since the P-DOM treatment involved GF/F filters throughout the incubation, "dummy" GF/F filters were added to the DOM and fulvic acid samples to create the same experimental conditions for all treatments. GF/F filters had been combusted at 450 °C for 2 h to remove organic materials and were then shredded into small pieces.

Inoculum, microbial species to induce biodegradation of organic matter, was prepared by filtering the downstream river water sample through a 2-μm polycarbonate membrane followed by incubating the filtrate in a rotary shaker for 7 days under aerobic conditions at 25 °C. The same amount of inoculum was added to each DOM, P-DOM, and fulvic acid sample at a concentration of 1% v/v (Fig. 1). Immediately after inoculum was added, part of the sample was filtered through a 0.2-μm polycarbonate

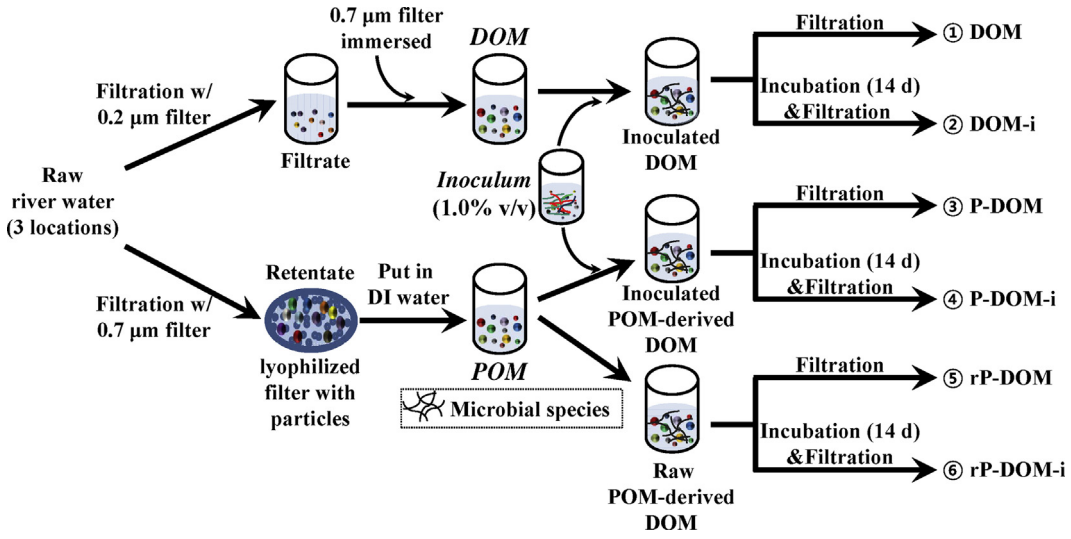

Fig. 1. Schematics of sample preparations. DOM and P-DOM samples were prepared from raw river water samples collected at three different locations (up-, mid- and downstream). Inoculum, microbial species added to induce biodegradation, was prepared from downstream river water by filtering through a 2-μm filter and incubating for 7 days. Incubation of DOM and P-DOM samples was carried out for 14 days at 25 °C followed by agitation at 150 rpm.

membrane filter to remove the inoculum, which was used as the 'before-incubation' sample. P-DOM samples were agitated on a reciprocal shaker for 24 h to leach soluble materials for determination of the initial DOC concentration. The "incubated" samples were obtained by filtering the inoculum-added solutions after 14 days of incubation at 25 °C in a rotary shaker under aerobic conditions. The container of each sample was opened for 30 min every 2–3 days in order to maintain aerobic conditions throughout the incubation. Since there was a possibility that some revived microbial organisms from freeze-dried microbial assemblies attached to sediment could degrade and transform labile fractions of P-DOM, P-DOM fractions without inoculum were treated using the same procedure as described above. Three replicate samples were prepared for each of the four treatments (DOM, P-DOM, raw P-DOM, and fulvic acid standard), resulting in a total of 20 samples.

2.2. AF4-UV-FLD analysis of organic matter

The AF4 channel utilized in this study was a model LC from Wyatt Technology Europe GmbH (Dernbach, Germany), with a tip-to-tip length of 26.6 cm, a thickness of 350 μm, and trapezoidal breadths of 2.2 cm and 0.6 cm at the inlet and outlet, respectively. The instrument was equipped with one of the following semipermeable membranes from Wyatt Technology: a polyethersulfone (PES) membrane or a Nadir regenerated cellulose membrane, both with a pore size of 5 kDa. The effective channel thickness was calculated as 306 μm from the experimental retention time and Stoke's diameter information of bovine serum albumin standard, and the calculated void volume of the channel was 0.95 mL. Carrier solutions used for AF4 separation were ammonium bicarbonate solution (ABC, pH 7.5) and phosphate buffered saline (PBS, pH 7.4) solution, and the concentrations of both solutions were varied at 2.0, 5.0, and 10 mM by dissolving adequate amounts of ABC powder purchased from Sigma (St. Louis, MO, USA) in ultrapure water (> 18 MΩ) and dilution of concentrated stock PBS solution from Sigma with ultrapure water. Each solution was filtered through two layers of nitrocellulose membrane (pore size of 0.22 μm) from Millipore (Danvers, MA, USA) prior to use.

A model SP930D HPLC pump from Young-Lin Instrument (Seoul, South Korea) was used to deliver carrier solution to the AF4 channel, and a model Standard 22 syringe pump from Harvard Apparatus (Holliston, MA, USA) was utilized for injection of the water sample. Sample injection to the AF4 channel was accomplished with a total of 2 mL of sample solution at a flow rate of 0.5 mL/min for 4 min while focusing flow streams (a total of 2.8 mL/min) were introduced from both the inlet and outlet channels at a 1:9 ratio for the focusing/relaxation process and simultaneous enrichment of sample components in the channel, as shown in Fig. S1 of Supplementary data. When the sample injection was complete, the focusing/relaxation process was allowed to continue for 1 min to assure relaxation had been achieved. Eluted sample components were monitored using a serially connected model M720 UV detector from Young-Lin at 254 nm and a RF-10AXL spectrofluorometric detector from Shimadzu Corp. (Kyoto, Japan) at two different combinations of fluorescence wavelengths, $\lambda_{ex}/\lambda_{em}$ (excitation/emission) of 275 nm/350 nm and 310 nm/412 nm. Both detector signals were recorded with Autochro-Win 2.0 Plus software supplied by Young-Lin.

2.3. EEM fluorescence spectroscopy of DOM fractions

To monitor the changes in optical properties of eluted components during AF4 separation, fractions of pre- and post-incubation organic matter samples were collected during elution based on UV detection, and fluorescence EEMs of the eluates were obtained with a model F-7000 fluorescence spectrophotometer from Hitachi High-Technologies Corp. (Tokyo, Japan). Excitation for EEM experiments was performed at 200–400 nm and emission at 290–540 nm with the following instrument parameters: scan speed of 2400 min^{-1}, response of 0.5 s and slit of 5 nm. A 290-nm cut-off filter was used to minimize secondary Rayleigh scattering. To partially account for Rayleigh scattering, the instrument response to blank runs with Milli-Q deionized water was subtracted from the EEMs of the samples. Blank runs were conducted for every batch of 10 samples. The Raman band intensity of water (Ex. 350 nm/Em. 400 nm) was used to monitor the stability of the instrument. The fluorescence intensities of samples were divided by the Raman band intensity in order to correct for any fluctuation in instrument conditions. Potential inner-filter effects of DOM absorption of incoming excitation light were corrected by applying a correction equation based on absorbance measurements at all wavelengths used to obtain fluorescence EEMs [39].

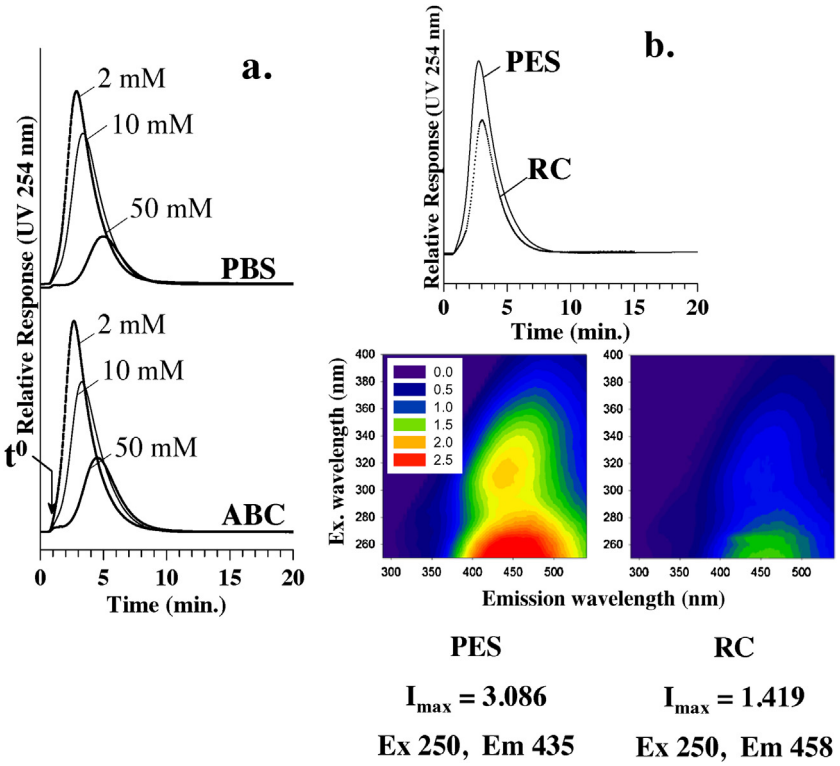

Fig. 2. Effects of (a) carrier solutions (PBS: phosphate buffered saline and ABC: ammonium bicarbonate) of varying ionic strengths (2, 10, and 50 mM) and (b) membrane materials (PES: polyethersulfone and RC: regenerated cellulose) on AF4 separation of a fulvic acid standard (pre-incubation), and EEM fluorescence spectra of the eluate collected during peak elution. EEM spectra were obtained from (a) each carrier solution (I = 2 mM) and (b) collected fractions of AF4 runs. Both membranes in run (b) had a MWCO of 5 kDa.

3. Results and discussion

Prior to the analysis of DOM and P-DOM samples, carrier solutions (PBS and ABC solutions) and membrane materials utilized in the AF4 channel system were evaluated using the Suwannee River fulvic acid standard at flow rate conditions of $\dot{V}_{out}/\dot{V}_c = 0.20/2.80$ (in mL/min). The sample injection rate was 0.50 mL/min, while focusing flows were delivered at a 1:9 (inlet:outlet) flow rate ratio and a total $\dot{V}_c = 2.80$ mL/min for 4 min. Fig. 2a compares the fractograms of fulvic acid standards at three different ionic strengths (2, 10, and 50 mM) in PBS and ABC solutions. It is apparent that retention of fulvic acid increased as ionic strength increased, resulting in significantly reduced signal intensity at a concentration of 50 mM for both PBS and ABC solutions. As the ionic strength increase, the electrical double layer at the surface of the channel membrane as well as organic matters would decrease, leading to a slower migration of sample components in the vicinity of the channel wall. However, as shown in Fig. 2a, more than 80% decrease in peak area was observed with the 50 mM solutions, which might be mostly caused by increased interaction between fulvic acid and the channel membrane. Since organic matter was collected from the AF4 for off-line EEM spectroscopic analysis, the background emission intensity of carrier solution needed to be considered. Upon examining EEM spectra of each carrier solution, it was found that 2 mM PBS solution exhibited a maximum peak intensity (I_{max}) in the EEM spectra of 0.953, which was less noisy than 2 mM ABC solution with I_{max} = 2.522. Therefore, 2 mM PBS solution was selected for the analysis of organic matter samples. While testing with fulvic acid standards, it was observed that the regenerated cellulose membrane (RC, 5 kDa) exhibited weaker peak signals compared to those with polyethersulfone (PES, 5 kDa), as shown in Fig. 2b, along with EEM spectra of the fulvic acid fractions collected during AF4 separation. EEM spectra of collected fulvic acid species showed fluorescence emission in the range of 400–500 nm, with a stronger peak intensity when PES was utilized as a channel membrane. This result indicates that there is considerable interaction between fulvic acid and the RC membrane, leading to some loss of analyte. Hence, the PES membrane was chosen for analysis of DOM and P-DOM samples.

Fig. 3 shows superimposed AF4 fractograms of pre- (solid line) and post-incubation (dotted) DOM, P-DOM, and rP-DOM samples from upstream, midstream, and downstream locations, based on humic-like FL detection at $\lambda_{ex}/\lambda_{em}$ = 310/412 nm. No sample exhibited any significant change in retention time caused by incubation, demonstrating that the size of the humic-like organic matter was not altered by incubation with microbial species. However, the fluorescence intensities of humic-like species in DOM, P-DOM, and rP-DOM samples from all three locations increased to some degree across the range of size fractions, with those for the midstream P-DOM sample showing about fivefold increase in peak area. An increase in peak area indicates that organic matter of similar size was generated in the process of uptake and release by microbial species. An earlier incubation experiment with water samples from the same sites in the Haean Basin and a downstream river in the Lake Soyang watershed [40] showed enhanced fluorescence in humic-like EEM peaks. Previous studies on biodegradable aquatic DOM [21,41,42] have provided supporting evidence that organic matter is not only consumed, but also produced by microbial species. Microbial processing of existing materials with various origins might result in a decrease in protein-like FL and enhancement in humic-like FL peaks associated with DOM moieties of either terrestrial or autochthonous origin [43,44]. The present study shows that organic matter from freshwater interacts with microbial species. Moreover, a significant change in peak area of the

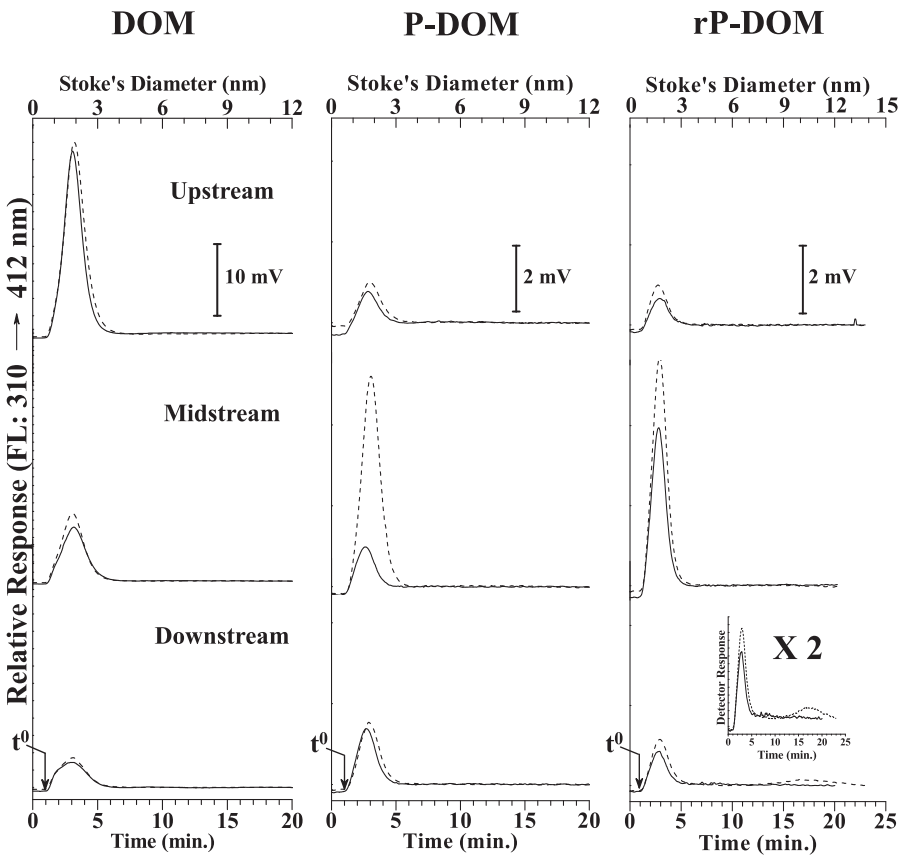

Fig. 3. Fractograms of DOM, P-DOM, and rP-DOM samples showing biodegradation induced by incubation. Fluorescence detection was performed at λ_{em} = 412 nm (λ_{ex} = 310 nm), representing humic-like signals. Dotted lines are for samples of post-incubation, while solid lines are for samples of pre-incubation. Signals of both downstream and upstream samples were magnified threefold for better comparison.

midstream P-DOM sample possibly reflects anthropogenic factors such as intensive agricultural land use that might significantly affect the organic matter composition and microbial community of the midstream freshwater [40]. Since agricultural land was in close proximity to the midstream location, heterogenic mixtures of organic matter could have been fluxed into the stream during preceding monsoon rainfall events, which may explain the unusual increase in the concentration of organic matter at this location.

Noteworthy was the appearance of an unknown peak in the incubated downstream rP-DOM sample in contrast to the non-incubated rP-DOM sample with no unknown peak after 20 min, as shown in Fig. 3 (a similar peak was observed in the UV fractogram shown in Supplementary Fig. S2). This was repeatedly observed over three runs. The calculated Stoke's diameter of the emergent peak was 10.65 nm, which was quite different from the sizes (<3.0 nm) of most of the OM species observed in this study as listed in Table S2 in Supplementary data. The emergent peak was collected for further analysis using MS and EEM, but the low concentration prevented the successful identification of the components. These emergent peaks might include large-sized, chromophoric and fluorescent organic matter, possibly created as by-products of microbial metabolism [45,46].

Biodegradation of organic matter in freshwater by microbial species is known to cause consumption and production of a considerable amount of humic-like organic matter in freshwater [40,44,47]. In order to evaluate the change in amount of organic matter, the peak areas of both DOM and P-DOM samples of pre- and post-incubation were compared using AF4-UV-FLD analysis. Fig. 4 shows changes in fractogram peak areas of incubated OM samples relative to the corresponding pre-incubation samples of fulvic acid standard, DOM, P-DOM, and rP-DOM samples. Bars in Fig. 4 represent relative changes in peak area based on (a) UV absorbance at 254 nm, representing the change in chromophoric species during incubation, (b) fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 310 nm/412 nm) for the change in humic-like species, and (c) fluorescence detection ($\lambda_{ex}/\lambda_{em}$ = 275 nm/350 nm) for protein-like species. Peak area values calculated from each fractogram are listed in Table S3 of Supplementary data. The relative peak areas of chromophoric organic matter as determined by UV detection varied little after the 14-day incubation for the fulvic acid standard and the three DOM samples, while the P-DOM and rP-DOM samples showed a wide range of increases in absorbance (20–760%) after incubation. Both rP-DOM samples from upstream and downstream, which were incubated with dormant microbial cells as part of freeze-dried sediment but not with inoculum, exhibited some increases in peak area that were either greater (up- and downstream samples) or smaller (midstream sample) than the corresponding P-DOM samples incubated with the inoculum. It should be noted that a significant production (7.6-fold increase) of chromophoric organic species was observed with the midstream P-DOM sample (see Table 1) after incubation. This data suggests that compared to upstream and downstream P-DOM samples, the midstream P-DOM sample contained various organic matter that is strongly influenced by incubation with inoculum. This result can be explained by the large quantity production of DOM from labile fractions of incubated particles by the activated microbial community of the added inoculum. The midstream P-DOM sample exhibited the highest rate of production of humic-like species (>5-fold relative to pre-incubation value), while the change in all of the DOM samples was relatively small. The direction of change in peak area after incubation clearly shows

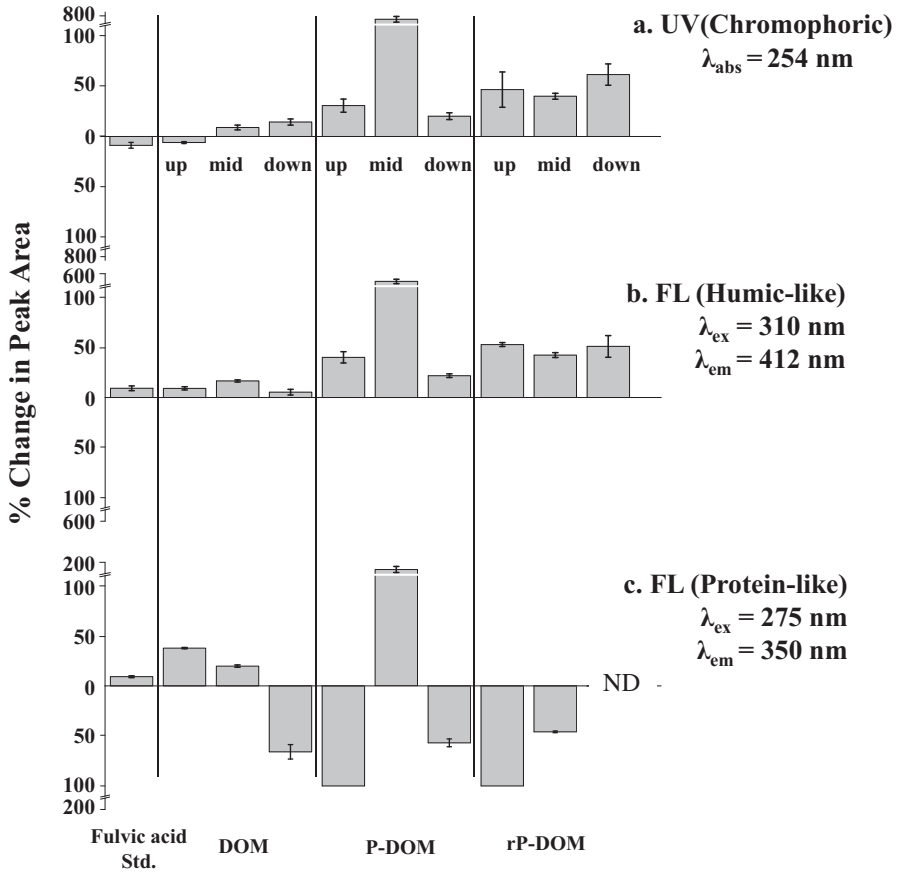

Fig. 4. Percent change in AF4 peak area after 14-day incubation of a fulvic acid standard, DOM, P-DOM, and rP-DOM samples based on UV detection at 254 nm (UV); FL detections classified as humic-like signals (excitation = 310 nm, emission = 412 nm) and protein-like signals (excitation = 275 nm, emission = 350 nm).

that chromophoric and humic-like fluorescent DOM components were more resistant to microbial degradation than the rather labile protein-like components of P-DOM and rP-DOM that could be transformed into humic-like species aided by microbial processing. For the protein-like species in Fig. 4c, both upstream and midstream DOM samples showed increases after incubation, but the downstream DOM and all of the P-DOM and rP-DOM samples except the midstream P-DOM exhibited considerable decreases. The significant decrease in concentration supports the biodegradation of labile protein-like components induced by incubation regardless of the presence of inoculum. However, the midstream P-DOM showed a large increase in protein-like species, presumably due to inoculum-enhanced conversion of other DOM fractions to protein-like moieties. Most of the P-DOM and rP-DOM samples showed increase in humic-like peak area after incubation, as shown in Fig. 4b.

The relative decrease in protein-like species and increase in humic-like species after incubation were observed in the EEM spectra of samples. Fig. 5 shows EEMs of the fulvic acid standard and the three DOM samples of pre-incubation (top row spectra) and post-incubation (middle) and the differential EEMs (bottom) of each sample during 14 days of incubation. Differential EEMs in the bottom row were obtained by subtracting the EEM spectra of the pre-incubation sample from those of each corresponding post-incubation sample. Fluorescence signal intensities were scaled using different colors for each set of samples. Since these results were based on the entire peak area over the range of wavelengths, they do not exactly represent the changes in concentration

Table 1
Relative changes in peak area of post-incubation OM samples.

		Chromophoric (%)	Protein-like (%)	Humic-like (%)
Std.	Fulvic acid	−8.78 ± 2.89	10.32 ± 0.85	10.20 ± 2.37
DOM	Up	−6.06 ± 0.99	38.54 ± 0.61	10.09 ± 1.59
	Mid	8.52 ± 2.35	20.81 ± 1.12	17.40 ± 1.05
	Down	13.88 ± 3.03	−66.35 ± 7.12	6.33 ± 2.82
P-DOM	Up	30.26 ± 6.51	−100.00 ± 0.00	40.74 ± 5.52
	Mid	764.00 ± 29.57	169.14 ± 13.14	477.32 ± 34.04
	Down	19.72 ± 3.36	−57.46 ± 3.85	22.61 ± 1.80
rP-DOM	Up	46.21 ± 17.55	−100.00 ± 0.00	53.35 ± 1.90
	Mid	39.59 ± 2.92	46.56 ± 0.81	42.95 ± 2.40
	Down	(1)	ND	(1)
		61.20 ± 10.56		51.51 ± 10.67
		(2) New peak		(2) New peak

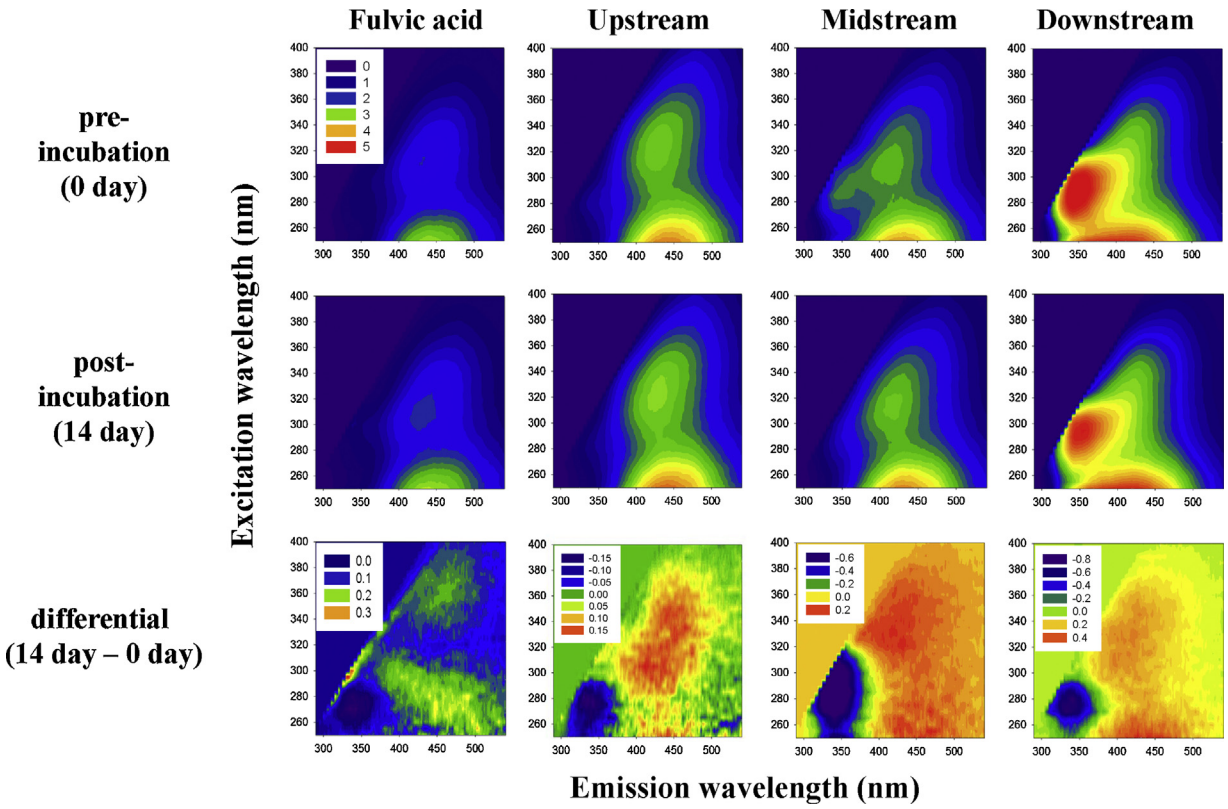

Fig. 5. Fluorescence EEM spectra of a fulvic acid standard and the three DOM samples of pre-incubation (top row), post-incubation (middle), and the differential EEMs (bottom) representing the net fluorescence signals (EEMs of 14 day – EEMs of 0 day).

observed in the analysis at each specific wavelength according to the AF4-UV-FLD system shown in Fig. 5. However, it is clear from the differential EEMs in Fig. 5 that protein-like species ($\lambda_{ex}/\lambda_{em}$ = 275 nm/350 nm) of both midstream and downstream samples decreased during incubation (dark blue for negative signals), while humic-like species ($\lambda_{ex}/\lambda_{em}$ = 310 nm/412 nm) gradually increased for those two samples, supporting that the conversion or production of humic-like species from microbial degradation of protein-like species. While EEMs demonstrate the overall changes in both chromophoric and fluorophoric species, the AF4-UV-FLD system provides specific information such as preferential uptake of certain types of organic matter or generation of different-sized organic matter.

4. Conclusions

This study demonstrated that asymmetrical flow field-flow fractionation can be utilized to elucidate the effect of metabolic activity of microbial organisms on the optical properties of DOM and P-DOM in multiple land-use watersheds, depending on size (DOM and P-DOM) and components (or composition) (humic-like and protein-like) of organic matter. Using AF4 combined with a serial connection of UV and FL detections as well as with EEM fluorescence spectroscopy, various chromophoric and fluorescent DOM fractions of a wide range of sizes can be simultaneously monitored at relatively high resolution. The on-line enrichment capability of the AF4 channel prior to separation for very low concentrations of organic matter in freshwater samples is quite useful since AF4 can enrich target materials in the vicinity of the channel membrane surface using a tangential flow. In addition, on-line enrichment and size separation/characterization of each organic matter sample can be accomplished within 30 min, which enables one to profile the changes in both size- and component-specific organic matter in relation to biodegradation. It is likely that humic-like DOM components derived from larger POM (>0.7 μm) are more susceptible to microbial activity than smaller DOM components (<0.2 μm), indicating the preferential consumption of labile organic matter by microbial organisms. Given the concurrent increase in the amount of large-sized humic-like DOM components when protein-like DOM components decreased during incubation compared to non-degraded sample, it is reasonable to assume that enhanced microbial activity consuming protein-like components contributed to formation of humic-like components of microbial origin. Although an exact mechanism of microbial consumption and production of organic matter requires further investigation, the AF4-UV-FLD system combined with off-line detection of fluorescence EEMs provides a powerful tool to identify size- and component-specific changes of DOM caused by microbial communities in various inland waters in a timely manner. Moreover, the AF4-UV-FLD system can be successfully applied to monitor changes in the quality and stability of freshwater DOM and P-DOM during transport to oceans or during water treatment processing.

Acknowledgment

This study was supported by a grant (2015R1A2A1A01004677) from the National Research Foundation (NRF) funded by the Korean government.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2015.07.074

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