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Photoalteration of dissolved organic matter (DOM) released from *Microcystis aeruginosa* in different growth phases: DOM-fraction distribution and biodegradability

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With 7 figures and 1 table

Abstract: The photoalteration of dissolved organic matter (DOM) produced in different growth phases of the blue-green alga, *Microcystis aeruginosa*, was investigated by comparing the biodegradability and distribution of fractions of algal DOM after different ultraviolet (UV) treatments. The distribution of DOM-fractions (based on hydrophobic-hydrophilic and acidic-basic breaks) showed that two of the fractions, hydrophilic acids (HiA) and bases (HiB), were more abundant in all growth phases of *M. aeruginosa* than the other three fractions, hydrophobic acids (HoA), hydrophobic neutrals (HoN), and hydrophilic neutrals (HiN). The proportion of HiB increased, while the HiA fraction decreased with aging of the algae. After UV treatment, all algal DOM became recalcitrant to bacterial degradation without complete photo-degradation. This was more pronounced in DOM from older cultures (stationary phase) as compared to DOM from the exponential growth phase. The DOM distribution was also significantly different after UV exposure, implying photoalteration to the chemical composition of algal DOM. The proportions of the HiB fraction decreased as a percent of the total dissolved organic carbon pool by 1.5–8.1 % after UVA treatment and by 5.3–15.8 % after UVB treatment. In contrast, the HiA fraction increased by similar amounts. Analyses of fluorescent properties and some carboxylic acids confirmed the changes to the HiB and HiA fractions. However, the increased HiA fraction may not be linked to the recalcitrance of algal DOM after UV exposure, since there was no difference in the biodegradability of this fraction before and after UV treatments. The ini-

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tially labile HiB fraction, however, became less available to bacteria after UV exposure depending on intensity and thus may be linked to the recalcitrance of algal DOM after UV exposure. Our results confirm earlier reports that algal DOM can be changed in its chemical composition as well as biodegradability by UV radiation, and suggest that the HiB fraction may be important in the formation of recalcitrant algal DOM.

Key words: algal DOM, growth phase, UV effects, photoalteration, biodegradability, chemical composition.

Introduction

Dissolved organic matter (DOM) can play a major role as a source of carbon for heterotrophic bacteria in freshwater ecosystems (WETZEL et al. 1972, AMON & BENNER 1994, CARLSON et al. 1994, LAMPERT & SOMMER 1997, WETZEL 2001). There are two major sources of DOM in lake waters: allochthonous, i.e. derived from the catchment area, and autochthonous, i.e. produced within lakes. Allochthonous DOM is composed of primarily terrestrial humic substances (HS) which are recalcitrant to bacterial degradation, while autochthonous DOM is composed of relatively more labile compounds (MUNSTER & CHROST 1990, WETZEL 2001). In most lakes, where allochthonous material is the dominant DOM source, most of the pool is comprised of recalcitrant DOM (WETZEL 2001). However, even in eutrophic lakes and oceanic waters, where the majority of DOM is autochthonous, much of the DOM pool is resistant to microbial degradation (SØNDERGAARD & MIDDELBOE 1995, CHOI et al. 2001).

Recent studies have noted that autochthonous DOM can be transformed into recalcitrant forms, without complete degradation to CO_2, after exposure to UV radiation, implying that photoalteration is manifest in chemical characteristics (TRANVIK & KOKALJ 1998, PAUSZ & HERNDL 1999, OBERNOSTERER et al. 2001). However, there is little information on the changes induced by UV radiation in the chemical characteristics of autochthonous DOM and, thus, a relevant approachr to evaluating photoalteration of algal DOM is needed. The first step may be to separate DOM into well-defined macromolecular fractions and to compare their distribution before and after UV exposure.

In pelagic waters, one of the most important sources of autochthonous DOM is extracellular organic matter (EOM) released from phytoplankton. This EOM may occur as a result of active excretion of photosynthetic products and/or leakage from senescent and dead algal cells, and its chemical composition varies with the physiological state of the algae (NALEWAJKO & LEAN 1972, SHARP 1977, FOGG 1982, CHROST & FAUST 1983, HAMA & HANDA 1987, BAINES & PACE 1991). It therefore seems reasonable to consider the physiological state of the algae in an examination of photoalteration of algal DOM.

The objective of this study was to examine the photoalteration of algal DOM produced from different growth phases of *Microcystis aeruginosa* by comparing the biodegradability and distribution of DOM-fractions, before and after UV exposure. The algal DOM was fractionated into five classes: hydrophobic acids, hydrophobic neutrals, hydrophilic acids, hydrophilic neutrals, and hydrophilic bases, using three kinds of resin adsorbents. To confirm the changes in the distribution of these fractions after UV exposure, we also examined their fluorescent properties and some of the organic acids of algal DOM. A bacterial degradation test was used as a measure of biodegradability of the algal DOM.

Materials and methods

Preparation of algal DOM

To obtain the algal-derived DOM, an axenic culture of *M. aeruginosa* (NIES-843), isolated from Lake Kasumigaura (Japan), was grown in ten litre (10 l) polycarbonate bottles at 25 °C and about 50 $\mu E\,m^{-2}/s^{-1}$ under a 12 h: 12 h light/dark cycle on CB medium. The culture was stirred by air bubbles from a pump equipped with a 0.2 µm sterilising filter. Since the standard CB medium contains a high concentration of organic carbon, we modified the medium by substituting K_2HPO_4 for B-glycerophosphate and $NaHNO_3$ for Tris buffer. The concentration of dissolved organic carbon (DOC) in the medium after inoculation was below 0.5 $mg\,l^{-1}$.

To determine the growth phases of the culture, its optical density (OD) was measured with a Shimadzu UV-2500 UV/VIS spectrophotometer at a wavelength of 550 nm using a 1 cm long quartz cell. The growth period was divided into one exponential and two stationary phases. When OD doubled within 24 h, growth was considered to be exponential (Phase I on day 7), and the following stages, in which OD remained more or less constant, were considered to be stationary phases (Phase II on day 10 and Phase III on day 13). In each growth phase, cultures were collected and then filtered through pre-combusted (450 °C for 4 h) Whatman GF/F glass-fibre filters. The filtrates were used as the source of algal-derived DOM.

UV treatments

For the UV treatments, triplicate 400 ml sub-samples of filtrate were transferred to 500-ml quartz tubes with silicon stoppers penetrated by three glass tubes. To estimate the effect of different UV radiation levels on the algal DOM, two artificial UV lamps were used throughout the experiments. UVB treatment (2.4 W/m^2 of UVB and 2.0 W/m^2 of UVA) was provided using two Philips TL 40 W/12 RS lamps with a wavelength range of 280 to 400 nm (maximum emission: 300 nm). UVA treatment (13.6 W/m^2 of UVA) was provided using four Q-Panel UVA-340 lamps (wavelength range: 300 to 400 nm, maximum emission: 340 nm) and UVB cutting film with zero transmission at 320 nm (C. I. Kasei, Japan). The quartz tubes containing the filtrate were irradiated for

24 h at 25 °C under the two different UV regimes. Sub-samples (20 ml) for analyses of DOC and fluorescence were taken at 2, 6, 12 and 24 h. All incubation during the UV treatments was conducted under sterile conditions by using a 0.2 µm sterilising filter. UV radiation was measured with a radiometer (MI-340 UV meter, Eikoseiki, Japan), equipped with a UV-A sensor (316–400 nm) and a UV-B sensor (280–315 nm).

Biodegradability experiments

The biodegradability of the algal DOM before and after UV exposure was quantified through a series of microbial degradation experiments. Before and after UV exposure a portion (200 ml) of each algal DOM sample was poured into pre-combusted 300-ml glass bottles (550 °C for 4 h), and 1 ml of bacterial concentrate was added to give an initial bacterial count of around 10^5 cells/ml. Water for the bacterial inoculum was collected from the hyper-eutrophic Furuike Pond, Japan. The bottles were then incubated in darkness at room temperature (ca 20 °C) for five days. Sub-samples (10 ml) for DOC determination were collected from the bottles after 0, 1 and 5 days. The biodegradability experiments were performed in triplicate.

DOM fractionation

Before and after the UV treatment and the biodegradation tests, the DOM samples were fractionated into five classes: hydrophobic acids (HoA), hydrophobic neutrals (HoN), hydrophilic acids (HiA), hydrophilic bases (HiB), and hydrophilic neutrals (HiN), based on their adsorption on to a series of macroporous resin adsorbents. The original fractionation method described by LEENHEER (1981) produced six fractions, including hydrophobic bases (HoB), but we disregarded the HoB fraction since it is known to be very small (IMAI et al. 1998).

Nonionic Amberlite XAD-8 resin (20–60 mesh), strong cation exchange resin (Bio-Rad AG-MP-50, 50–100 mesh), and strong anion exchange resin (Bio-Rad AG-MP-1, 50–100 mesh) were used for the fractionation. The column capacity factor, k′, for separating hydrophobic acids through the XAD-8 resin column was 50. Appropriate classification of organic compounds according to the DOM fractions is listed in Table 1 (LEENHEER 1981, THURMAN 1985).

The XAD-8 resin was cleaned and conditioned as described by THURMAN & MALCOLM (1981). Three millilitres (3 ml wet volume) of the XAD-8 resin was packed into a glass column and rinsed three times, alternating from 0.1 M NaOH to 0.1 M HCl, just

Table 1. Classification of organic solutes for dissolved organic carbon (LEENHEER 1981, THURMAN 1985).

Fraction	Solute compound classes
hydrophobic acids (HoA)	humic substances (humic and fulvic acids)
hydrophobic neutrals (HoN)	hydrocarbons, carbonyl compounds
hydrophilic acids (HiA)	carboxylic acids (fatty and hydroxyl acids), sugar acids
hydrophilic bases (HiB)	protein, amino acids, aminosugars
hydrophilic neutrals (HiN)	oligosaccharides, polysaccharides

before application of the sample. A blank sample was collected in the final rinse with 0.1 M HCl (B1). Both AG-MP-50 (hydrogen-form) and AG-MP-1 (chloride-form) resins were Soxhlet-extracted with methanol for 24 h. AG-MP-1 was then converted into the free base-form with 1 M NaOH and rinsed with Milli-Q water (Milli-Q SP. TOC, Millipore). Glass columns containing 6 ml (wet volume) of the cation exchange resin and 12 ml (wet volume) of the anion exchange resin were connected in series and conditioned by pumping about one litre of Milli-Q water through the resins. Blank samples (B2 and B3) were collected from each column after conditioning.

The flow scheme of the DOC fractionation procedure was as follows (IMAI et al. 1998).

Step 1: Acidify filtrates (DOC1) to pH 2.0 with 6 M HCl, pass 200 ml of the filtrate through the XAD-8 column by a peristaltic pump with Tygon tubing at a flow rate of about 1 ml/min, and rinse the column with 1–2 bed volumes of 0.1 M HCl.

Step 2: Elute the column in the reverse direction with more than 3 bed volumes of 0.1 M NaOH at a flow rate not exceeding 0.5 ml/min (DOC2), and measure the elutant volume.

Step 3: Pump the effluent from the XAD-8 column (DOC3) through a series of cation-anion resin columns at a flow rate of about 1 ml/min, and after pumping 1–2 bed volumes of the sample, collect elutant samples from the anion resin column (DOC5) and then from the cation resin column (DOC4).

DOC fractionation was performed in duplicate. After the fractionation, DOC was measured for DOCs 1–5 and for the blank samples. Each DOC fraction was calculated as follows:

$$\mathrm{HoA} = \mathrm{DOC2} \times (\text{elutant volume})/(\text{sample volume}) \quad (1)$$

$$\mathrm{HoN} = (\mathrm{DOC1} - \mathrm{B1}) - \mathrm{HoA} - \mathrm{DOC3} \quad (2)$$

$$\mathrm{HiB} = (\mathrm{DOC3} - \mathrm{B1}) - (\mathrm{DOC4} - \mathrm{B2}) \quad (3)$$

$$\mathrm{HiA} = (\mathrm{DOC4} - \mathrm{B2}) - (\mathrm{DOC5} - \mathrm{B3}) \quad (4)$$

$$\mathrm{HiN} = \mathrm{DOC5} - \mathrm{B3} \quad (5)$$

In order to examine the recovery efficiency of the DOM fractionation method, the relative standard deviation (RSD) of the duplicated measurement for each fraction was estimated according to Standard Methods (APHA 1998). The RSD values were less than 10 % for the determination of the HoA, HiA and HiB fractions and 20 % for the HoN and HiN fractions.

Chemical analyses

Some carboxylic acids that were found to be major products formed during UV exposure (BERTILSSON & TRANVIK 1998, WETZEL 2000), were analysed on a capillary ion electrophoresis (CIE) system (Quanta 4000E, Waters). Two millilitres of each algal DOM sample were collected in polypropylene vials and 20 µl of octansulfonate was added to a final concentration of 70 µM to obtain isotachophoretic conditions during the electromigrative sample introduction (30 s at 5 kV). Duplicate sub-samples of 0.5 ml were added to polypropylene vials for analysis. A 60 cm fused silica capillary

(75 µm inner diameter), and a 100 mM sodium boric acid buffer containing 0.5 mM of an electro-osmotic flow modifier (OFM-BT, Waters) were used for the analysis. A separation voltage of 10 kV was applied and detection of carboxylic acids was accomplished by indirect UV detection at 185 nm. Standard curves (10–500 µg/l) were made for the three carboxylic acids (oxalic, formic, and acetic acids) detected.

Fluorescence can provide rapid and sensitive analysis of DOM, such as humic-type and protein-like DOM (MAYER et al. 1999). In the present study, we measured the fluorescence at 270/350 nm of excitation/emission because the excitation/emission wavelength is used as an index of protein-like DOM in natural water and phytoplankton exudates (MAYER et al. 1999, FUKUSHIMA et al. 2001). A fluorescence spectrophotometer (Shimadzu RF-5300 PC) equipped with a 150 W xenon lamp was used for the fluorescence measurements. The fluorescence of Milli-Q water was used as a blank.

DOC was measured as non-purgeable DOC with a Shimadzu TOC-5000A total organic carbon analyser equipped with Pt catalyst on quartz wool. Triplicate measurements were made for each sample and analytical precision was within 1 % of the coefficient of variance. Potassium hydrogen phthalate (Kanto Chemical Co., Tokyo) was used as the standard.

Results

Photoalteration of algal DOM produced from *M. aeruginosa*

Exposure to UV radiation made no significant changes to the amounts of dissolved organic carbon (DOC) in the DOM produced from different growth

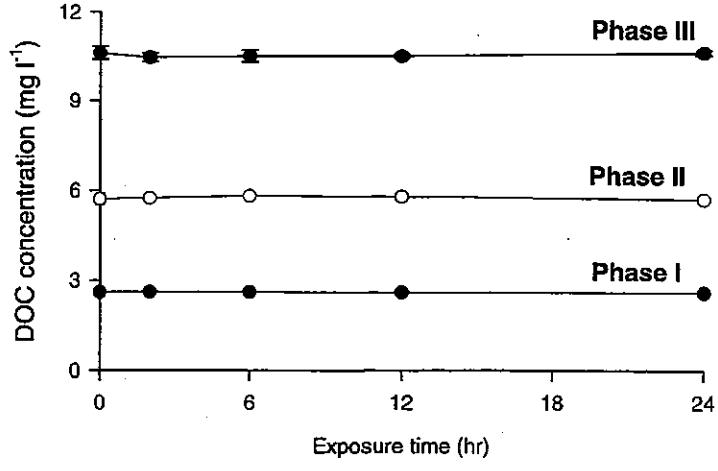

Fig. 1. Changes in DOC concentrations of algal DOM exposed to UV for various lengths of time (Phase I: exponential growth phase on day 7, Phase II: stationary phase on day 10, Phase II: stationary phase on day 13). Error bars represent the standard deviation of the mean of triplicate treatment flasks (Errors less than the size of the symbols are not shown).

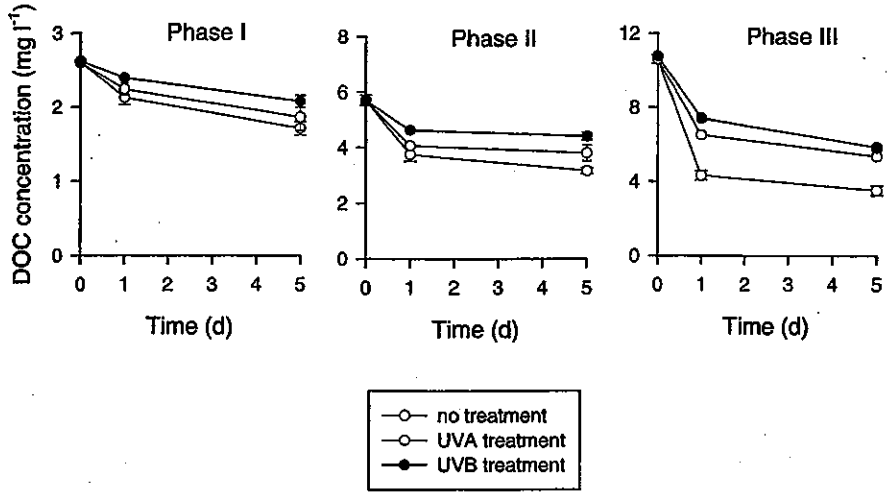

Fig. 2. Decomposition curves for algal DOM obtained from different growth phases and after UV treatment. Error bars represent standard deviations of means of triplicate treatment flasks (Errors less than the size of the symbols are not shown).

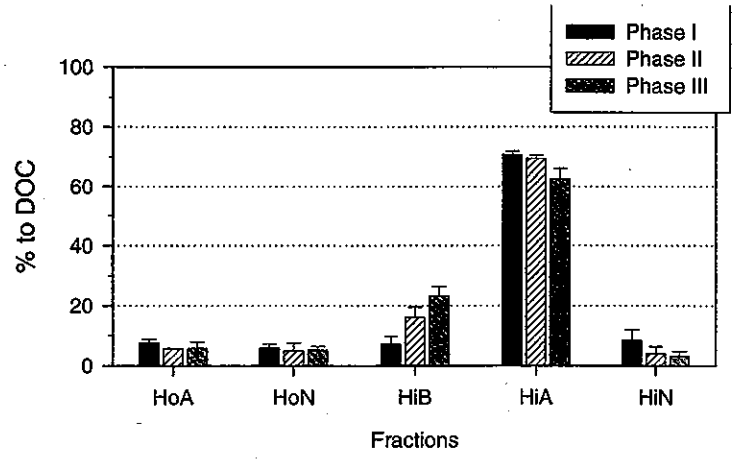

Fig. 3. Fractions of DOM released by *M. aeruginosa* in different growth phases (HoA: hydrophobic acids; HoN: hydrophobic neutral; HiB: hydrophilic bases; HiA: hydrophilic acids; HiN: hydrophilic neutrals). Error bars represent standard deviations of means of duplicate fractionation for each fraction.

phases of *M. aeruginosa*. Levels remained constant at 2.61 ± 0.01 mg/l in Phase I, 5.75 ± 0.05 mg/l in Phase II and 10.53 ± 0.07 mg/l in Phase III (Fig. 1). In contrast, exposure to UV made a considerable difference to the biodegradability of the algal DOM (Fig. 2). Biodegradability decreased after exposure,

depending on the UV treatment, and the decrease was largest in the oldest culture. For example, the microbial degradation (for 5 days) of the DOM from growth phase III decreased by up to 17.4 % after UVA treatment and by 21.1 % after UVB treatment, when compared to no UV treatment.

DOM-fraction distribution of algal DOM

Hydrophilic fractions of the DOM predominated in all growth phases of *M. aeruginosa* (Fig. 3). In particular, the HiA fraction was found to be the most abundant in all growth phases, representing 62.6–70.7 % of the algal DOM. The HiB fraction was the second most prominent, accounting for 7.2–23.2 %. Thus, the HiA and HiB fractions are likely to be the most significant fractions produced by *M. aeruginosa*. With aging of the algae, the proportions of the HiB fraction increased, while the HiA and HiN fractions decreased. The hydrophobic fractions (HoA and HoN) were consistently a minor proportion of the total in all growth phases, together accounting for less than 13 % of the algal DOM.

After exposure to UV radiation, however, the composition changed depending on the growth phase and the UV treatment (Fig. 4). The proportions of the HiB and HiA fractions were considerably changed compared to the other fractions. In all growth phases the proportion of the HiB fraction decreased after UV exposure (by 1.5–8.1 % after UVA treatment and by 5.3–15.8 % after UVB treatment). In contrast, the HiA fraction increased by as much as the decrease in the HiB fraction after UV exposure (by 4.7–8.7 % after UVA treatment and 9.3–16.3 % after UVB treatment). The changes in proportions of the two fractions after UV radiation increased with the age of the culture.

Evidence of photoalteration in two fractions, HiA and HiB, of algal DOM

To clarify the changes in the two fractions (HiB and HiA) after UV exposure, we measured the fluorescence at 270/350 nm, as an index of protein-like DOM for the HiB fraction, and several carboxylic acids for the HiA fraction.

The fluorescence (at 270/350 nm) of the algal DOM (whole DOM before fractionation) was high in Phase III (1.61) and low in Phase I (0.13) (Fig. 5 a). After exposure to UV, the fluorescence declined exponentially with UV exposure time in all growth phases, and the decrease was marked in Phase III after UVB treatment (Figs. 5 a, b).

Three carboxylic acids (oxalic, formic and acetic acids) were detected in all the growth phases of *M. aeruginosa* (Fig. 6). Oxalic acid occurred in low concentrations (below 10 µg/l in all phases) and showed little variation with the age of the culture. In contrast, formic and acetic acids had relatively high

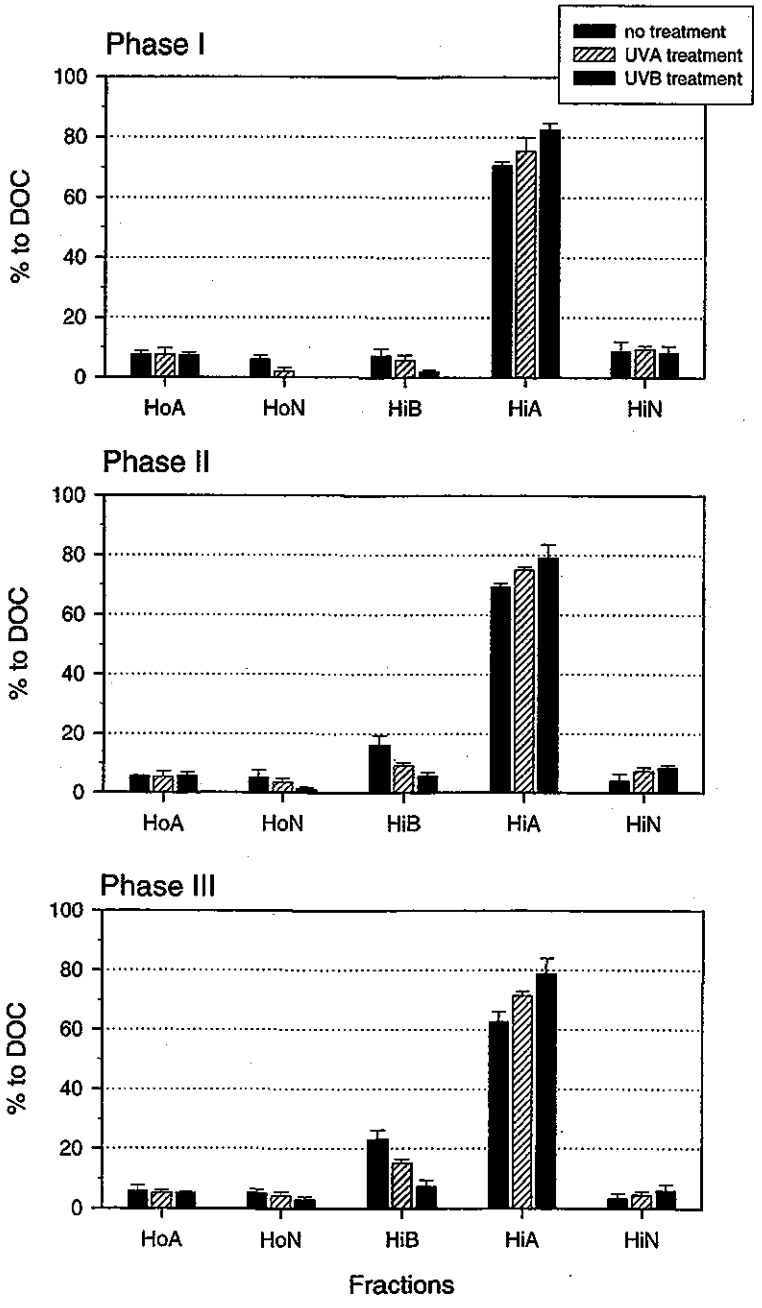

Fig. 4. Fractions of algal DOM obtained from different growth phases and after UV treatment (HoA: hydrophobic acids; HoN: hydrophobic neutral; HiB: hydrophilic bases; HiA: hydrophilic acids; HiN: hydrophilic neutrals). Error bars represent standard deviations of means of duplicate fractionation for each fraction.

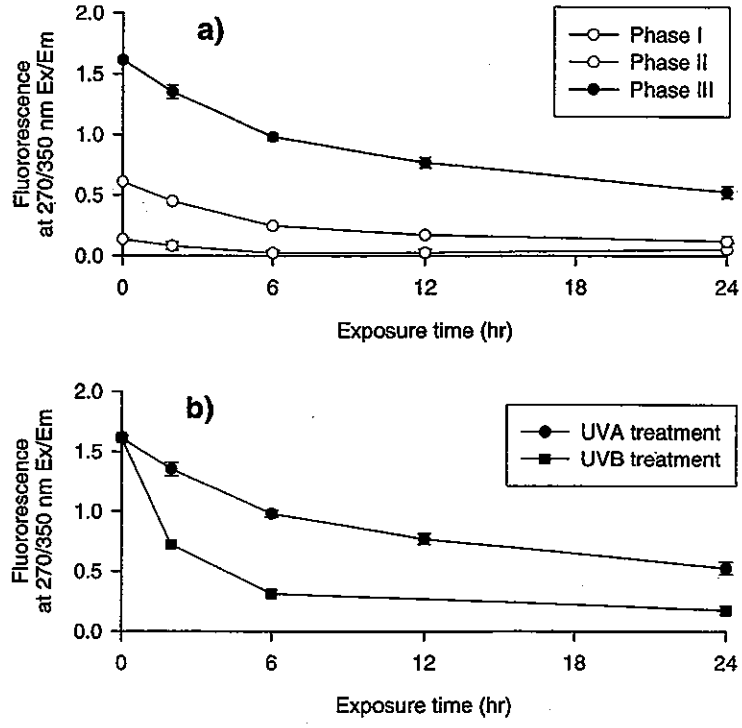

Fig. 5. Changes in fluorescence at 270/350 nm (Ex/Em) of algal DOM exposed to UV for various lengths of time; (a) for growth phases I, II and III after UVA treatment, (b) for Phase III algal DOM after UVA or UVB treatment. Error bars represent standard deviations of means of triplicate treatment flasks (Errors less than the size of the symbols are not shown).

concentrations (33–226 µg/l and 18–206 µg/l, respectively), and increased greatly with the age of the culture (see white bars in Fig. 6). After UV treatment, the three carboxylic acids greatly increased in all the DOM sources, indicating photochemical production of carboxylic acids from algal DOM. The increase in carboxylic acids was higher after UVB treatment than after UVA treatment in samples from all growth phases. For example, compared to no UV treatment, acetic acid increased up to 72 µg/l after UVA treatment and up to 153 µg/l after UVB treatment in Phase II.

Decreased biodegradability of HiB fraction after UV treatment

A different biodegradability (measured as the percentage of DOC utilized compared to the initial DOC in the fraction) was observed in the HiB fraction after UV treatment (Fig. 7). The HiB fraction produced by *M. aeruginosa* was initially very labile to bacterial degradation, showing a high biodegradation of

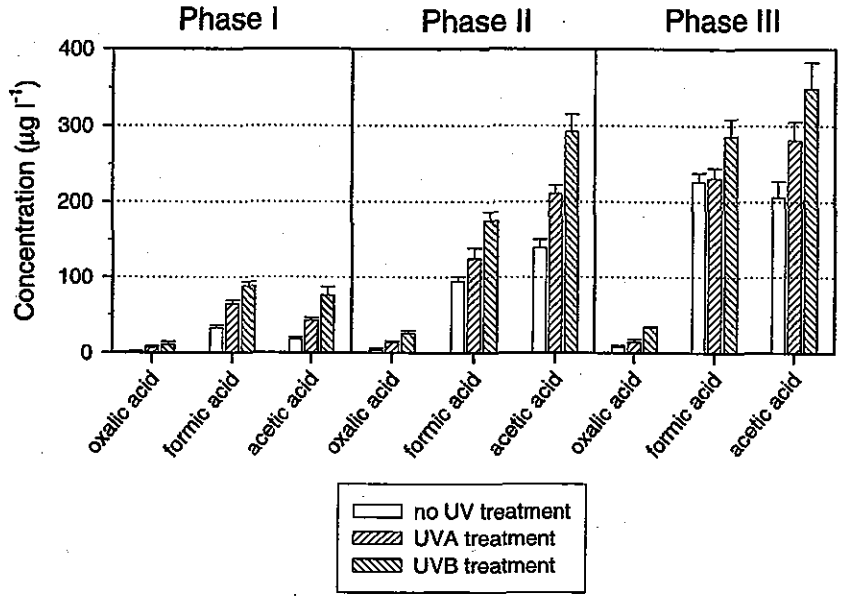

Fig. 6. Concentrations of three carboxylic acids in algal DOM obtained from different growth phases and after UV treatment, indicating UV-induced increase of oxalic, formic and acetic acids. Error bars represent standard deviations of means of triplicate treatment flasks.

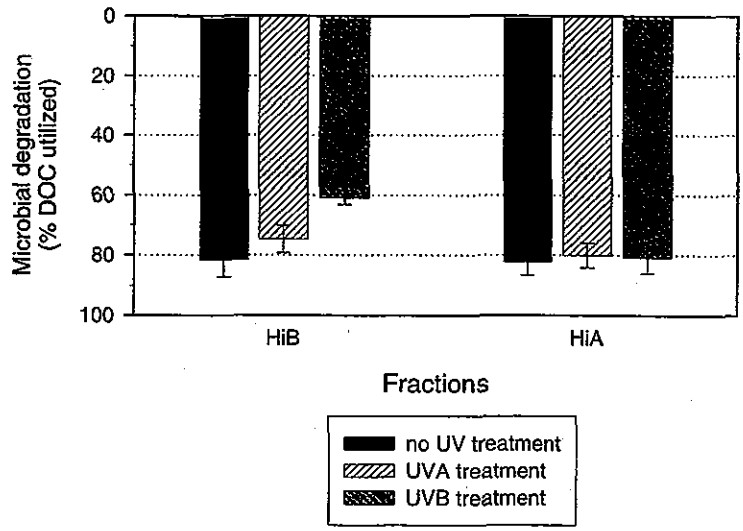

Fig. 7. Microbial degradation of the HiB and HiA fractions in algal DOM from Phase III before and after UV treatments. Samples were incubated in darkness at room temperature for 5 days. Error bars represent standard deviations of means of triplicate treatment flasks.

81.6 ± 5.6 % over 5 days. However, this biodegradability decreased significantly after UV treatment ($p = 0.009$ for UVA treatment and $p = 0.007$ for UVB treatment by paired t-test), and the decrease was greater in the UVB treatment than in the UVA treatment (74.7 ± 4.6 % after UVA treatment and 60.8 ± 2.3 % after UVB treatment). On the other hand, there was no difference in biodegradability of the HiA fraction between before and after UV treatments (Fig. 7).

Discussion

It is well known that UV radiation can alter the DOM pool by causing complete degradation into CO_2, and by cleaving the DOM into smaller and more labile molecules, enhancing bacterial activity (Miller & Zepp 1995, Wetzel et al. 1995, Amon & Benner 1996, Moran & Zepp 1997, Gardner et al. 1998, Kieber et al. 1999, Wetzel 2000). However, the extent of photochemical transformation of DOM into CO_2 shows a wide range from 0 to 60 % in many natural waters (Wiegner & Seitzinger 2001). Clear photo-oxidation has been observed only in waters containing high levels of humic substances (HS), but with no or little algal DOM. Complete photo-oxidation may be limited to allochthonous DOM, high in HS, because HS strongly absorb short wavelength light (Frimmel 1994), and most HS are not derived from algae but rather higher plants (Wetzel 2001). In addition, recent studies have shown that initially labile algal-derived DOM becomes more recalcitrant after UV exposure (Tranvik & Kokalj 1998, Pausz & Herndl 1999). These studies found that microbial activity in DOM which had been exposed to UV was inhibited by 15 to 20 %, while the loss of DOC was less than 1 % during UV exposure. Research to date has shown that the effects of UV radiation depend largely on the DOM source as well as the light source and length of exposure. In the present study, all the algal DOM produced from different growth phases of *M. aeruginosa* was transformed into more recalcitrant forms after UV exposure without photo-oxidation (Figs. 1 and 2). These results confirm several recent findings on the decreased biodegradability of algal DOM due to UV radiation, and indicate that these findings are common in algal DOM.

Furthermore, there was a difference in the distribution of DOM-fractions after UV exposure, especially in the two major fractions (HiB and HiA) of DOM produced by *M. aeruginosa* (Fig. 4), reflecting photoalteration in the fractional composition of algal DOM as well as the biodegradability. The changes in the two fractions after UV radiation were clear in the oldest culture. After UV exposure, the HiB fraction decreased, while the HiA fraction increased by as much as the decrease in HiB. In contrast, Thomas & Lara (1995) showed that algal DOM was not changed in chemical composition or

concentration after UV exposure. The difference between their results and ours may be due to the sources of algal DOM used in the two studies. We used freshly produced algal DOM, while the DOM used by THOMAS & LARA (1995) had been aged in the presence of bacteria for 8 months. During this long incubation, bacteria would utilise initially labile constituents that could be changed by UV radiation. Thus, initially labile DOM was not involved in their experiments despite the fact that they are important fractions of algal DOM.

The classification of organic solutes by several researchers has suggested that the HiB and HiA fractions consist mainly of protein-like and carboxylic acid-like DOM, respectively (Table 1). Although the specific organic compounds of the HiB fraction were not identified in this study, the fluorescence at 270/350 nm of excitation/emission, used as an index for the protein-like DOM, supports the decrease in the HiB fraction due to UV radiation (Fig. 5). In addition, the increase of three carboxylic acids after exposure to UV supports the increase of the HiA fraction. Several studies suggest that the photochemical formation of carboxylic acids is linked to the presence of humic substances (ALLARD et al. 1994, BERTILSSON & TRANVIK 1998, WETZEL 2000). In this study, on the other hand, the photochemical production of carboxylic acid-like fractions may be related to the non-humic fractions of algal-derived DOM. However, the increased HiA fraction may not be linked to the recalcitrance of algal DOM caused by exposure to UV, since carboxylic acids are, in general, easily taken up by bacteria (BERTILSSON & TRANVIK 1998), and since the HiA fraction in this study was shown to have high biodegradability after UV treatment (Fig. 7).

Some studies indicate that labile proteinaceous substrates could be transformed into recalcitrant forms during UV exposure (NAGANUMA et al. 1996), or only after a long incubation (KEIL & KIRCHMAN 1994). In this study, the proportion of HiB increased with aging of the algae (Fig. 3), and the decreased biodegradability of algal DOM was more marked in the older phases (Phase II and III) than in the exponential phase (Phase I) (Fig. 2). Furthermore, the initially labile, protein-like HiB fraction became increasingly recalcitrant to bacterial degradation after exposure to UV (Fig. 7). These results indicate that the protein-like fraction may be important in the decrease of biodegradability of algal DOM by UV exposure.

The present study indicates that algal DOM can be photochemically altered in its chemical composition and biodegradability, and the photoalteration may be more important in older phases than in the exponential growth phase of the algae. Furthermore, our results suggest that the protein-like HiB fraction may be important in the formation of recalcitrant DOM.

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水環境におけるフミン物質の特徴と役割

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特集

水環境における腐植物質の役割と分析法の進歩

土壌などに含まれる有機成分である腐植（フミン）物質は，植物が腐朽してできた物質や微生物の代謝物などが複雑に反応してできた天然の高分子物質である。最近では，腐植（フミン）物質が環境中に放出された化学物質の運命に大きく関与している可能性が明らかにされつつあり，その機能が注目されている。本特集では，水環境において腐植（フミン）物質がどのような役割を演じているかについて概説するとともに，その挙動および特性の解明に向けた最新の分析方法の紹介と，土壌浄化を中心とした環境修復への利用について紹介する。（担当編集企画委員　独立行政法人産業技術総合研究所・市川廣保，兵庫県立健康環境科学研究センター・駒井幸雄）

水環境におけるフミン物質の特徴と役割*

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1．はじめに

フミン物質（腐植物質，humic substances）は水環境や陸上環境に遍在的に存在する，濃縮すると黄色から黒色を呈する，生物起源で不均質な難分解性の有機物である[1]。フミン物質は土壌有機物の主要な構成要素であり，植物や微生物への養分供給や土壌の団粒構造の保持等の機能を担っている。このためフミン物質の研究は土壌フミン物質を対象として開始され，今日まで2世紀に渡って行われてきた。

一方，水環境でのフミン物質の研究は，湖沼，河川，地下水に溶存しているフミン物質（溶存態フミン物質，aquatic humic substances）が対象であったため，技術的な困難さから，その研究の本格的な進展は米国地質調査研究所の研究者によって定量的分離手法が開発された1980年代初めまで待たねばならなかった[2]。当時，溶存フミン物質の研究が急速に進展した背景要因の一つは，フミン物質を含む水道原水を塩素殺菌処理した場合に有害な消毒副生成物が産生されることが明らかになった[3]ためと言われている。

本稿では，水環境中において溶存態として存在するフミン物質を対象として，その定義，特性（濃度・存在比，分子量等）や水環境における役割・機能について概説する。一般に，フミン物質（humic substances）は，酸やアルカリへの溶解性によって，フミン酸（酸不溶，アルカリ溶解），フルボ酸（酸とアルカリに溶解）およびヒューミン（酸とアルカリに不溶）に操作的に分類される。すなわち，本稿で対象とするフミン物質とは溶存態のフミン酸とフルボ酸ということになる。これ以降，本稿では，溶存態のフミン物質を“フミン物質”と称する。

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2．水環境中のフミン物質の特徴

2.1　フミン物質の定義

土壌フミン物質の定義が操作的なものであるように[4]，水環境中のフミン物質も実験操作的に定義された

ものである。天然水中からフミン物質を分離するために幾つかの手法が提案されてきたが，現時点で最も一般的なものは非イオン性樹脂(XAD-8等)を用いた吸着クロマトグラフィーによる分離手法と言える。この手法はフミン物質の持つ疎水性的性質と酸性官能基含有量に基づいたものである。ThurmanとMalcolm[2]は，そのランドマーク的論文において，pH2で，カラム容量ファクター100の条件下で，50%がXAD-8樹脂カラムに吸着し，0.1MのNaOHで溶出するものをフミン物質（aquatic humic substances）と定義した。フミン物質のうち，濃度500mgC・l^{-1}以上，pH1で沈殿するものをフミン酸，溶存しているものをフルボ酸とした。この分離手法は国際腐植物学会(IHSS)[5]で推奨されている手法でもある。ただし，近年，カラム容量ファクターとして100ではなく50という値が使用されている[6,7]。

XAD-8樹脂吸着クロマトグラフィーによるフミン物質の分離手法には，落とし穴が一つある。肝心のXAD-8樹脂が生産中止となり樹脂の入手が極めて困難なことである。同じアクリルエステル系のXAD-7樹脂は市販されているが，0.1MNaOHでフミン物質を樹脂から溶出させると大量の溶存有機物が樹脂から漏れ出してしまう[8]。最近XAD-8樹脂の代替としてDAX-8樹脂(スペルコ社製)が使用され始めている。DAX-8樹脂は，少し吸着能が高いが，フミン物質分離に関してXAD-8樹脂とほとんど差はないようである[9]。

2.2 フミン物質の濃度と存在比（図1）

フミン物質の濃度は水環境によって異なる[10]。一般に地下水や海水では非常に低い(0.05−0.60mgC・l^{-1})。渓流水・河川水や湖水では0.5−4.0mgC・l^{-1}，泥炭地や湿原水では10−30mgC・l^{-1}と非常に濃度が高い。このセクションでは，様々な水環境中のフミン物質の濃度や存在比について概説する。ここではフミン物質の組成にはほとんど言及しないが，存在する水環境が著しく異なるとフミン物質の組成・特性も顕著に異なることは既に認識されている[11]。

(a) 河川水・渓流水

米国における渓流水・河川水の溶存有機物（dissolved organic matter, DOM）の主要コンポーネントはフミン物質であり，平均でDOMの約50%を占めると報告されている[10]。河川水フミン物質のほとんどはフルボ酸であった（80-90%）。存在比に関する例外的ケースは，泥炭地や湿原を水源とする茶褐色を帯びた河川水である。例えば，湿原を水源とする米国スワニー河[7]や熱帯域に位置するブラジル・リオ・ネグロ川[12]では，DOMの70-90%はフミン物質である。湿原水中のフミン物質の存在比は非常に高いようである。サロベツや霧多布の湿原水を調査したところ，フミン物質の存在比は実際とても高かった（50-75%，今井未発表データ）。

わが国の河川水や渓流水中のフミン物質の存在比に関する報告例はそれほど多くない。琵琶湖北湖に流入する4河川水のフミン物質は，DOMの37-73%（0.32-0.71 mgC・l^{-1}）を占めていたと報告されている[13]。DOM濃度が最も低い森林自然系河川水（安曇川）でフミン物質の存在比が最大となった。また霞ヶ浦に流入する主要4河川水のフミン物質は0.43-1.39mgC・l^{-1}で，その平均存在比は38.6%（31.1-42.7%）であった[14]。

(b) 地下水

地下水は表流水に比べてフミン物質の含有率が少ないようである。地下150m以深の地下水では，フミン物質はDOMの12-33%であったとの報告がある[10]。CronanとAiken[15]は，浅いO/A層土壌水では，フミン物質はDOMの約46%占めたが，深いB層土壌水では27%で

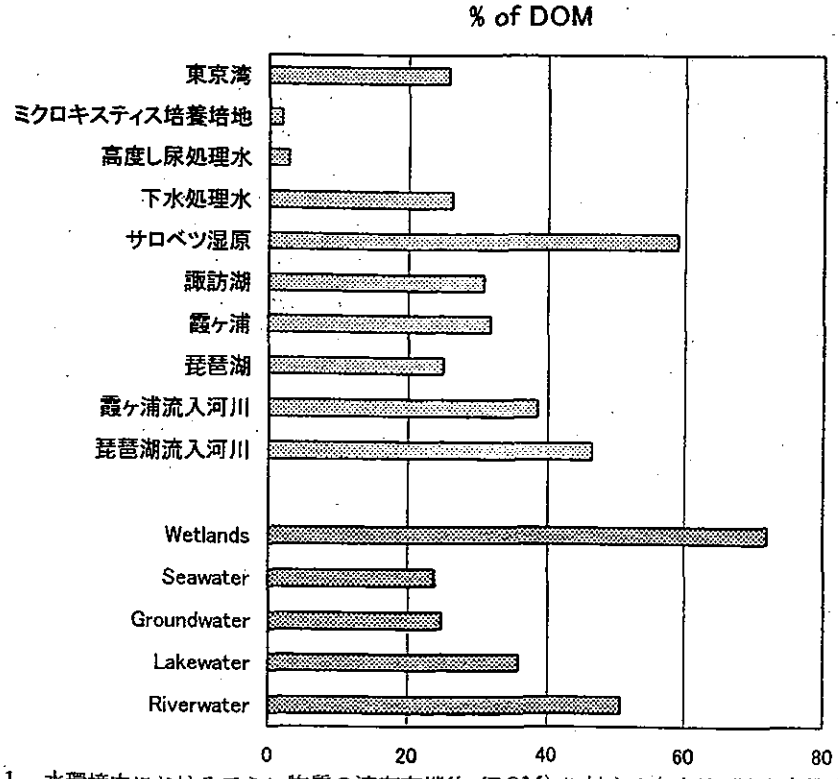

図1 水環境中におけるフミン物質の溶存有機物（DOM）に対する存在比（溶存有機炭素DOCとして）。英文表記は国外データを示す。

あったと報告している。存在比の深さ方向の減少は無機土壌によるフミン物質の選択的吸着のためと説明された。

(c) 湖　水

湖水フミン物質の存在比は，一般に，DOMの40%程度と言われている[10]。フミン物質の濃度は湖の栄養状態が高まると増大する（貧栄養湖：0.5-1.0，中栄養湖：1.0-1.5，富栄養湖：1.5-5.0mgC・l^{-1}）。河川水と同様に，湖水でもフルボ酸がフミン物質として優占する(85-90%)。

湖水フミン物質は外部由来フミン物質と湖内部で生産されるフミン物質の混合物と考えられる。外部由来と内部生産由来フミン物質の相対的な寄与率は，湖沼の物理的サイズや主要流入DOM源が土壌由来か，河川由来か，あるいは藻類由来かに関係している[10]。一般に，湖沼のサイズが大きいほど，藻類由来DOMの湖沼有機物プールへの寄与が大きくなる。ところが，藻類由来DOMに占めるフミン物質の割合はとても低い。Choiら[16]は3種の藍藻類を室内培養して培地中のフミン物質を測定したところ，その存在比は0.2%－16%の範囲にあったと報告している。すなわち，湖のサイズが大きいほど，藻類由来有機物が主要なDOM源であるほど，湖水フミン物質の存在比は低くなる傾向にある。

わが国における湖水フミン物質に関する情報を調べてみると，その存在比は琵琶湖北湖で約25%[13]，霞ヶ浦で32%[14]，諏訪湖で31%，手賀沼で30%（今井未発表データ）であった。琵琶湖でフミン物質の存在比が比較的低いのは，琵琶湖が他の湖沼に比較してサイズがとても大きく，DOM源としても藻類由来DOMが優占しているためと推測される。

(d) 海　水

海洋においてフミン物質はDOMの5%-25%(0.06-0.60mgC・l^{-1})を占めている[17]。そのほとんど(>90%)はフルボ酸である[18]。Fukushimaら[19]は広島湾のDOMの33% (0.40mgC・l^{-1})はフミン物質であったと報告している。東京湾表層水ではDOMの25-28%をフミン物質が占めていた（今井未発表データ）。外洋中に蓄積されているDOMのほとんどは藻類等の微生物由来と考えられる[20]。すなわち，陸地から離れるほどフミン物質のDOMに対する寄与は低下するだろうと推察される。

(e) 排水処理水

Imaiら[21]は様々な排水処理水中のフミン物質濃度を測定した。フミン物質の存在比は下水処理水で18-27% (0.6-1.1mgC・l^{-1})，し尿処理水で3-24% (0.1-8.7 mgC・l^{-1})，合併処理浄化槽排出水で28% (1.7mgC・l^{-1})であった。下水処理水では処理場の規模が大きくなるほどフミン物質の存在比が大きくなった。限外沪過膜等を採用している高度し尿処理場処理水にはフミン物質はほとんど含まれていなかった。

2.3 分子量と分子構造

(a) 分子量

分子量とサイズ分布に関する情報は，フミン物質のバルク的な物理化学的特性を理解するうえでとても重要である。Thurmanら[22]は，小角X線散乱法により，水環境中のフルボ酸の分子量は500－2,000，フミン酸の分子量は10,000以下であり，当時考えられていた分子サイズよりもかなり小さいことを示した。フルボ酸は同じようなサイズの分子の集合体で，一方，フミン酸は著しく分子サイズが異なる分子の集合体であることも指摘された。Chinら[23]は，無関係電解質の溶離液への適正な添加とランダムコイル状の非タンパク質ポリマー（polystyrene sulfonates）を分子量スタンダードとして用いることにより，高速液体サイズ排除クロマトグラフィーによってフミン酸やフルボ酸の分子量を高い信頼性をもって測定できることを示した。水環境中に存在するフミン物質（主にフルボ酸）の分子量は概ね1,000－2,000の範囲にあることが示された。

(b) 分子構造

Leenheerら[24]は，IHSS標準サンプルである米国スワニー河フミン物質を対象とした研究成果を基に，フルボ酸に対する幾つかの構造モデルを提案した。フルボ酸は基本的に不均質であり，同じフルボ酸分子でも反応性に顕著な違いのあることが指摘された。MacCartyとRice[25]は，生態学的な観点から，フミン物質の持つ特性，(1)分子構造の不規則性と(2)微生物が関与する特異的な生成経路の欠如は，フミン物質の共通的な特徴である難分解性（残存性）と遍在性を説明していると主張した。つまり，規則正しい順序で配列できないモノマー(単量体)の複雑で不均質な混合物であるフミン物質は，微生物がその進化の過程において，わざわざ特異的な酵素を誘導して分解するには余りにも利益の少ない炭素基質とみなせる。従って，微生物に無視されたフミン物質は，難分解性物質として残存し続けてきたと言える。

一方，最近の分析機器の進展に伴い，これまで得ることができなかったフミン物質の分子レベルの構造特性がかなり明らかとなってきた。Leenheerら[26]は，電子スプレーイオン化多段式質量分析計によって，スワニー河フルボ酸に対する単一の前駆化合物の存在を示し，その構造を提案している。

3．水環境中におけるフミン物質の役割・機能

水環境中におけるフミン物質の役割は，基本的に“modifier”と表現されるような，何らかの作用を緩衝・修正する機能を担っていると考えられる。同時に，その機能は二元的(正と負)，環境条件によって効果が逆転するような側面を持っている。

3.1 毒　性

フミン物質の毒性に関する情報は極めて少ないが，人の健康に直接関係しているとする2，3のケースが報告されている。Luら[27]は，台湾において，井戸水中のフミン物質と黒足病との因果関係を示唆している。Pengら[28]は，飲料水中のフルボ酸がカシンベック（Kasin-Beck）病におけるフリーラディカル生成メカニズムに直接的に関与すると報告している。

3.2 トリハロメタン生成能

フミン物質が浄水処理プロセスの塩素殺菌過程において産生される発ガン物質トリハロメタン等の消毒副生成物の主要な前駆物質であることは良く知られている[3,29]。フミン物質は，凝集沈殿や活性炭吸着の浄水処理によって良好に除去できるようである[30]。最新の研究では，フミン物質の存在比が低い水源では，フミン物質よりも非フミン物質（親水性DOM）の方がトリハロメタン