Michael U. Kumke, Fritz Hartmann Frimmel

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2.11 Stationary and time-resolved fluorescence for humic substances characterization

M. U. Kumke[1] and F. H. Frimmel[2]

Institute of Physical Chemistry and Theoretical Chemistry, University of Potsdam, Karl-Liebknecht-Str. 24-25, 14476 Golm, Germany

Engler-Bunte-Institut, Division of Water Chemistry, University of Karlsruhe, Engler-Bunte-Ring 1, 76131 Karlsruhe, Germany

Steady-state and time-resolved fluorescence methods were applied to investigate the fluorescence properties of humic substances of different origins. Using standard 2D emission and total luminescence spectra, fluorescence maxima, the width of the fluorescence band and a relative fluorescence quantum efficiency were determined. Different trends for fulvic acids and humic acids were observed indicating differences in the heterogeneity of the sample fractions. The complexity of the fluorescence decay of humic substances is discussed and compared to simple model compounds. The effect of oxidation of humic substances on their fluorescence properties is discussed as well.

2.11.1 Introduction

The importance of understanding the interaction mechanism between humic substances and xenobiotics has already been pointed out. The ultimate goal is to use fast and simple measurements for an identification of HS and an estimation of their interaction with different xenobiotics in order to predict fate and transport of these chemicals for a fast and reliable risk assessment. Together with other analytical

techniques, spectroscopic approaches have been widely used for the investigation of humic substances (HS) and their environmental-relevant reactions. In particular, fluorescence spectroscopy has been applied for the characterization of HS because of its high selectivity and its outstanding sensitivity. The non-invasive character of the experiments and the capabilities to monitor reactions on a sub-nanosecond time-scale make it highly attractive for the investigation of HS and reactions of HS with xenobiotics. In the priority program ROSIG a joined effort was made to increase the understanding of those reactions. The scope of the work presented here was a thorough investigation of the intrinsic fluorescence properties of HS. A part of the work is closely related to results presented in Chapter four “molecular interactions” of this textbook, particularly to the contributions of Löhmannsröben et al. and Kopinke et al.

HS are a complex, heterogeneous mixture of compounds originating from degradation of plant and animal tissues. The question of existence and the search of a general structure of HS seem therefore somewhat ill-defined. Although a general structure of HS can not be proposed, a classification scheme of HS and of their reactions with xenobiotics is within reach. This scheme can be based on the information on the precursor materials, on the origin of HS and on their characteristic properties. However, a major drawback in HS research results from the extreme heterogeneity of the samples and the information on HS gained by analytical techniques suffers strongly from this fact. The application of fluorescence spectroscopy as analytical technique can overcome several of the limitations and yield useful information on structure and reactivity of HS. In fluorescence spectroscopy multidimensional measurements including variations of the excitation wavelength λ_{ex}, emission wavelength λ_{em}, fluorescence lifetime τ_f and fluorescence anisotropy ρ can be used to gain specific information on complex mixtures like HS.

To reach this aim, one has to understand the processes connected with the intrinsic fluorescence properties of HS and has to identify the most important fluorescence parameters needed to address the issues under investigation.

Based on considerations of precursor materials and on degradation experiments, common structural features of HS have been suggested (Lanvik at al. 1994, Leenheer et al 1995a, Leenheer et al. 1995b, Liao et al. 1982, Schulten 1995, Schulten et al. 1987, Senesi et al. 1991). The importance of specific substructures as a potential reaction sites for metal ion complexation and in the formation of hazardous compounds (e.g., desinfection-by-products) has also been discussed. The importance of aromatic moieties in the formation of MX during water desinfection was investigated (Langvik et al. 1994). However, experimental approaches using pyrolysis or other intrusive techniques are prone to artifacts. The application of non-invasive techniques such as fluorescence that have already been used for the determination of metal binding to HS or the determination of acid-base properties of HS, seems much more promising (Casassas and Tauler 1995, Da Silva and Machado 1994, Da Silva et al. 1996). Recently, fluorescence spectroscopy, in particular synchronous fluorescence and total luminescence, have been successfully used to distinguish between HS isolated from soil and aquatic origins (Da Silva and Machado 1997, Mobed et al. 1996, Patterson et al. 1992, Pullin and Cabaniss 1995, Senesi et al. 1989) or to determine the concentration of HS in natural fresh waters (Mittenzwey et al. 1996, Hautala et al. 2000). Fluorescence spectroscopy and especially fluorescence quenching have been applied as powerful tools for the investigation of interactions and transformations of organic xenobiotics in the presence of HS (Kumke et al. 1994, Zimmermann et al. 1997, Illenseer et al. 1999, Doll et al. 1999, Kumke et al. 2000a, Kumke et al. 2000b). A more detailed

discussion of these issues is beyond the scope of the work discussed here and is presented in detail in Chapter four of this textbook.

The time course of the fluorescence of HS following pulsed excitation has been investigated using time-resolved fluorescence techniques in the time-domain as well as in the frequency-domain (Power et al. 1986, Cook and Langford 1995, McGown et al. 1995, Zimmermann et al. 1997). A highly complex fluorescence decay was reported for the HS investigated. In the data evaluation different models were applied, however only little effort has been spent in a detailed interpretation of the obtained data.

Although, some limitations caused by the heterogeneous character of HS have to be accepted, the outstanding sensitivity and selectivity make fluorescence a method of choice for the investigation of humic substances and their reactions. However, further knowledge is required for a thorough understanding of the intra- and intermolecular processes relevant to the fluorescence of HS. The objectives of the work were to gain deeper insight in the intrinsic processes connected to the fluorescence of HS and to identify suitable parameters for a meaningful interpretation of these phenomena on the molecular level. Ultimately, based on these parameters it was to develop classification scheme for HS applicable for the prediction of environmentally relevant reactions. Therefore, the first step is to elucidate the processes directly and indirectly influencing the intrinsic HS fluorescence.

2.11.2 Experimental section

In this study the fulvic acid (FA) fractions and humic acid (HA) fractions of a brown water (HOx, x = 10, 13, 14), of a soil seepage water (BS1), of a ground water (FG1),

of a waste water effluent (ABVx, x =2, 3), and of a production effluent from brown coal industry (SV1) were investigated. The isolation procedure and basic characterization data for these samples are described in detail elsewhere (Frimmel and Abbt-Braun 1999). FA and HA stock solutions were diluted to a final concentration of 10 mg/L. For the determination of the fluorescence efficiencies, the optical densities of the samples and of the salicylic acid reference were adjusted to 0.1 to achieve equal absorption conditions and to minimize inner filter effects (IFE). The pH-value of the samples was adjusted to seven using a standard phosphate buffer. The ionic strength of the samples was approx. 0.02 M.

The fluorescence experiments were carried out using a FL/FS900CDT combined fluorescence lifetime spectrometer (Edinburgh Analytical Instruments, UK). The instrument was equipped with a 450 W Xenon arc lamp (steady-state operation), a pulsed nitrogen flash lamp (time-resolved mode), and red-sensitive photomultiplier tubes which were operated in the single photon counting mode. The instrument is described in detail elsewhere (Kumke et al. 1998a, Kumke et al. 1998b). Because of the extremely heterogeneous character of the HS samples an absolute determination of fluorescence quantum yields could not be done in a straightforward manner. Therefore, the quantum efficiencies relative to the fluorescence of salicylic acid were determined and are further referred to as relative fluorescence quantum efficiencies (RFQE). For the determination of the quantum efficiencies, the fluorescence spectra of the HS samples were recorded in the wavelength range of 300 nm < λ_{em} < 600 nm using an excitation wavelength λ_{ex} = 295 nm.

The total luminescence was determined in the wavelength range of 275 nm < λ_{ex} < 401 nm ($\Delta\lambda$ = 3 nm) and 281 nm < λ_{em} < 545 nm ($\Delta\lambda$ = 1 nm). The spectra were recorded with a spectral bandpass of 1.8 nm for the excitation as well as the

emission monochromator. All fluorescence spectra were corrected for instrumental response functions (e.g., quantum efficiency of the photomultiplier tube) using a calibration function provided by Edinburgh Instruments. For the determination of the relative fluorescence quantum efficiencies (RFQE) spectral bandpasses of 1 nm were used in the excitation and emission path. The dwell time of the measurements was set to 0.5 s.

2.11.3 Results and discussion

2.11.3.1 Steady-state fluorescence of HS

In general, the fluorescence of HS is measured as a featureless, broad-banded spectrum with emission maxima approx. between 420 nm < $\lambda_{em,\ max}$ < 480 nm. Unlike the absorption spectra, which show a continuous increase from low absorbance in the NIR to high absorption in the UV, the fluorescence excitation spectra exhibit a maximum between 315 nm < λ_{ex} < 370 nm. However, it has to be emphasized that the fluorescence intensity and the shape of the fluorescence spectrum of humic substances are strongly dependent on experimental parameters. Due to the presence of a continuous absorption spectrum with gradually increasing absorbance to the UV region the induced inner filter effects are wavelength-dependent. This applies for the excitation as well as the emission wavelengths used. Furthermore, the fluorescence efficiency and the shape of the fluorescence spectrum are strongly dependent on the excitation wavelength. The observed fluorescence spectrum also dependent on parameters of the solution of which the pH is probably the most important one to be considered.

In table 2.11.3-T1 the fluorescence maxima and the width of the emission band are compared. In the measurements an excitation wavelength of λ_{ex} = 295 nm was chosen. The spectra were corrected for the spectral response of the detector. After converting the spectra from the wavelength scale to the wavenumber scale, it was found that all spectra were almost perfectly gaussian shaped. A data analysis using a mono modal gaussian distribution function yielded fits with correlation coefficients r^2 > 0.98. The band width of the emission spectra of HA fractions was found to be broader than the related FA fraction. Although HA are considered of larger size with a

higher content of aromatic structures this was not reflected in the steady-state spectra in terms of the location of the emission maxima.

Table 2.11.3-T1

HS[a]	**RFQE[1] in %**	**emission maximum[2] in cm^{-1}**	**width of emission band[2] in cm^{-1}**
ABV2 FA	4.7	22800	7801
ABV2 HA	2.8	22889	9104
ABV3 HA	5.1	22225	8407
SV1 FA	1.8	22760	7409
SV1 HA	2.8	23880	7576
FG1 FA	9.4	21948	6918
FG1 HA	6.4	20942	7499
BS1 FA	3.0	21920	6609
BS1 HA	2.1	22177	7429
HO10 FA	3.0	21513	6929
HO10 HA	1.6	21275	7900
HO13 FA	3	21981	6748
HO14 FA	1.9	21879	6902

[a]The fluorescence measurements were performed with an excitation wavelength λ_{ex} = 295 nm and an optical density at λ_{ex} of 0.1.
[1]Relative fluorescence quantum efficiency (RFQE) determined relative to the fluorescence of salicylic acid which was set to 100%
[2]determined by a gaussian fit of the emission spectrum

While in the location of $\nu_{em,\ max}$ of the FA only small differences were observed, the $\nu_{em,\ max}$ of HA showed larger variations (see table 2.11.3-T1). In order to reduce the number of parameters that influence the fluorescence spectrum, the total luminescence (TL) spectra were recorded.

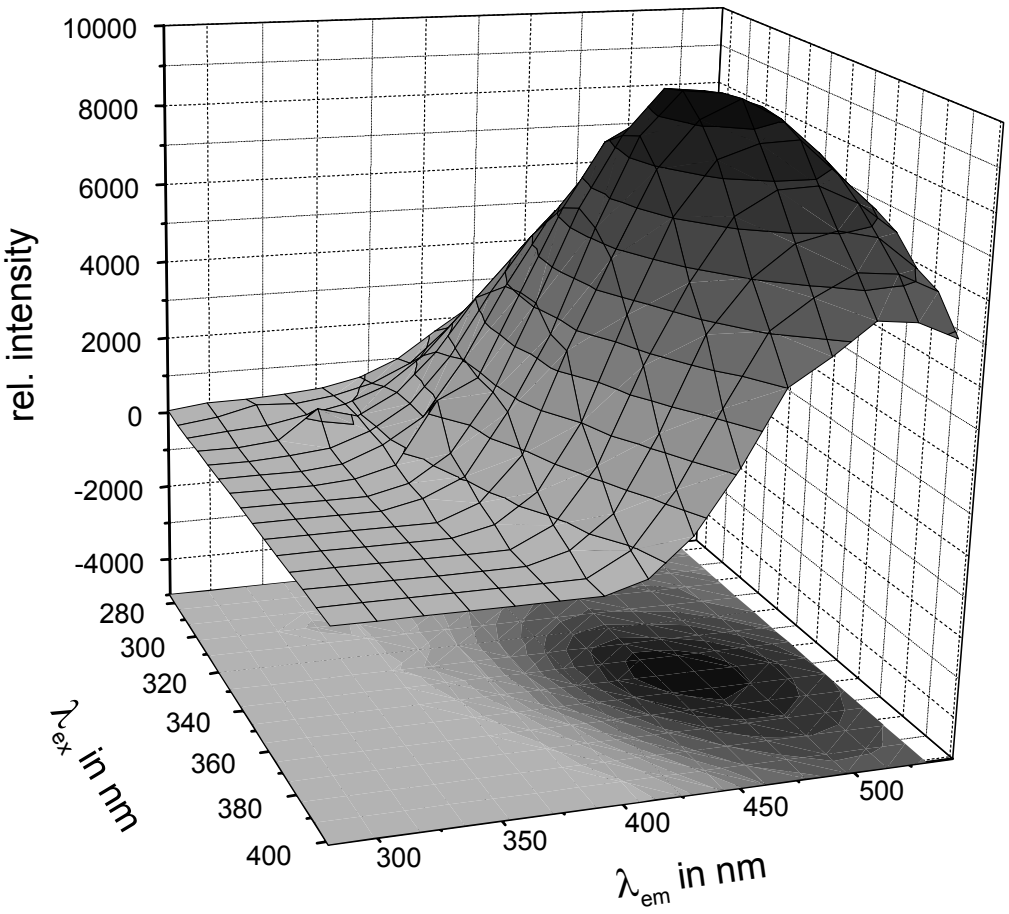

Figure 2.11.3-F3

Correlation of the fluorescence emission maximum $\nu_{em,max}$ (with λ_{ex} = 295 nm) with the ratio of the absorption at λ = 254 nm and λ = 203 nm.

In figure 2.11.3-F1 the TL spectrum of a brown water FA (HO14 FA) is shown. In the upper part of the figure the 3D representation is shown and in the bottom part a 2D projection in the form of a contour plot of the same spectrum is given. The increase in the color intensity corresponds to the increase of the fluorescence intensity. In the TL spectrum, the influence of the excitation wavelength on the observed fluorescence can be seen. This can be used as an additional selection parameter (Mobed et al. 1996). Since the excitation wavelength was changed during the experiment and the absorption spectra of the HS samples were different, the adjustment to a constant

pre-selected optical density was not applicable. Instead, the concentration of dissolved organic carbon (DOC) was adjusted to 10 mg/L for all samples and the pH was set to 7. From the obtained TL spectra excitation / emission pairs were obtained for maximum fluorescence intensity. For all samples investigated the $\lambda_{ex,max}$ / $\lambda_{em,max}$ data pairs are shown in Figure 2.11.3-F2.

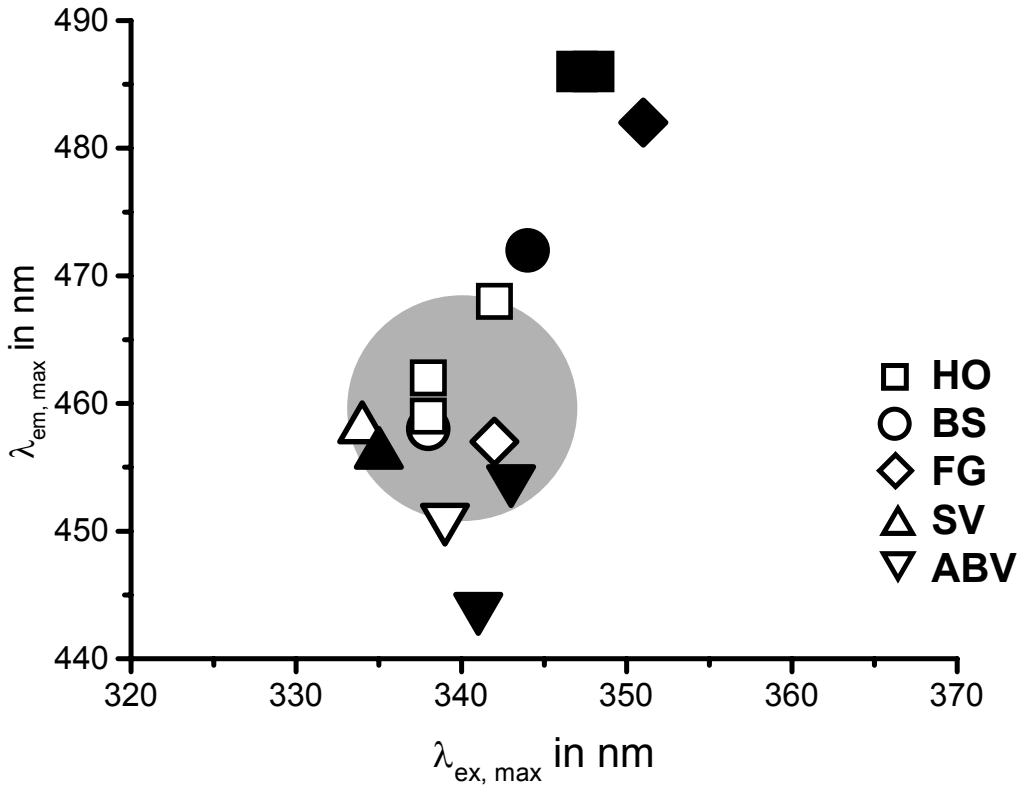

Figure 2.11.3-F2
Wavelength pairs of $\lambda_{ex,max}$ and $\lambda_{em,max}$ for HA (filled symbols) and FA (open symbols) fractions determined from the corresponding TL spectra (DOC = 10 mg/L, pH 7).

Similar to the data already discussed with the 2D spectra, in the TL measurements of the HA fractions a relatively large variation in the position of the wavelength pair corresponding to the maximum fluorescence intensity was observed, while on the

other hand, only a very small variation was found for the FA fractions. For all the FA investigated the $\lambda_{ex,max}$ / $\lambda_{em,max}$ data pairs were located in a small wavelength range of $\Delta\lambda_{ex}$ ~ 15 nm and $\Delta\lambda_{em}$ ~ 15 nm (the grey area in figure 2.11.3-F2).

The fluorescence quantum yield is also a useful parameter for the identification of a substances and for the characterization of interactions with its molecular environment. For HS in general a determination of a quantum yield is ill-defined due to the heterogeneous character of the material. However, a relative quantum efficiency under well-defined experimental conditions can be obtained and used for the comparison of different humic substances. The determination of a relative fluorescence quantum efficiency (RFQE) has the advantage that it is independent of the instrument used and therefore, data acquired in different laboratories could be easily compared. To circumvent the tedious calibration with fluorescence standards a simple relative measurement with salicylic acid used as a reference compound was performed. The optical density of all samples was adjusted to 0.1 at λ = 295 nm and the quantum efficiency was determined relative to salicylic acid which was set as 100 %. In table 2.11.3-T1 the RFQE of the HA and FA investigated are compared. Compared to that of salicylic acid, the fluorescence quantum efficiencies of all HS samples were quite low with an average value of approx. 3 %. Significantly higher values were found for the ground water samples FG1 FA and FG1 HA. As a general trend, it was found that the FA had a slightly higher RFQE compared to the related humic acids.

According to Korshin et al. the absorption at 203 nm and at 254 nm can be ascribed to the benzenoid (Bz) and electron-transfer (ET) bands of aromatic compounds (Korshin et al. 1997). It was proposed that due to the presence of polar functional groups (e.g., carbonyl, carboxyl, and ester groups) attached to the aromatic units the intensity of the ET band can be

affected to a large extent while the intensity of the Bz band is much less sensitive. On the other hand, non-polar groups were not presumed to affect the ET band. In HS the vast majority of groups active in fluorescence can be considered of benzenoid character (Senesi et al. 1991). The fluorescence of benzene is weak, but it can be enhanced in the presence of attached functional groups, like hydroxyl or carboxyl groups. Hence, a correlation of the fluorescence change and the degree of substitution with functional groups obtained from absorption experiments was investigated. In figure 2.11.3-F3 a correlation of the observed location of the fluorescence maximum (λ_{ex} = 295 nm) with the ratio of the Bz and ET bands is shown.

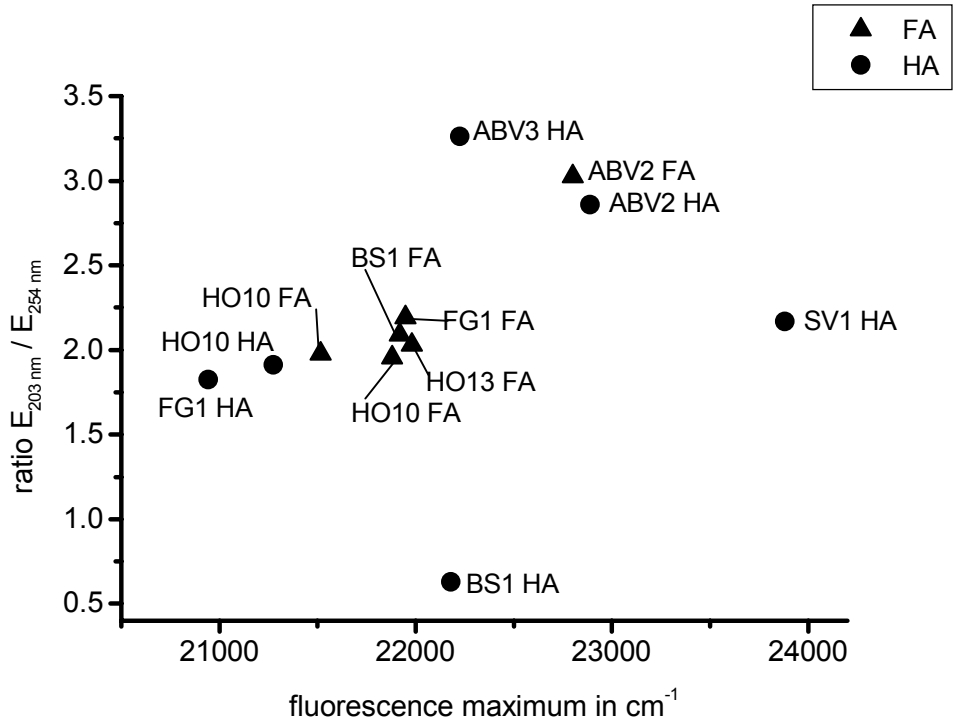

Figure 2.11.3-F3

Correlation of the fluorescence emission maximum $\nu_{em,max}$ (with λ_{ex} = 295 nm) with the ratio of the absorption at λ = 254 nm and λ = 203 nm.

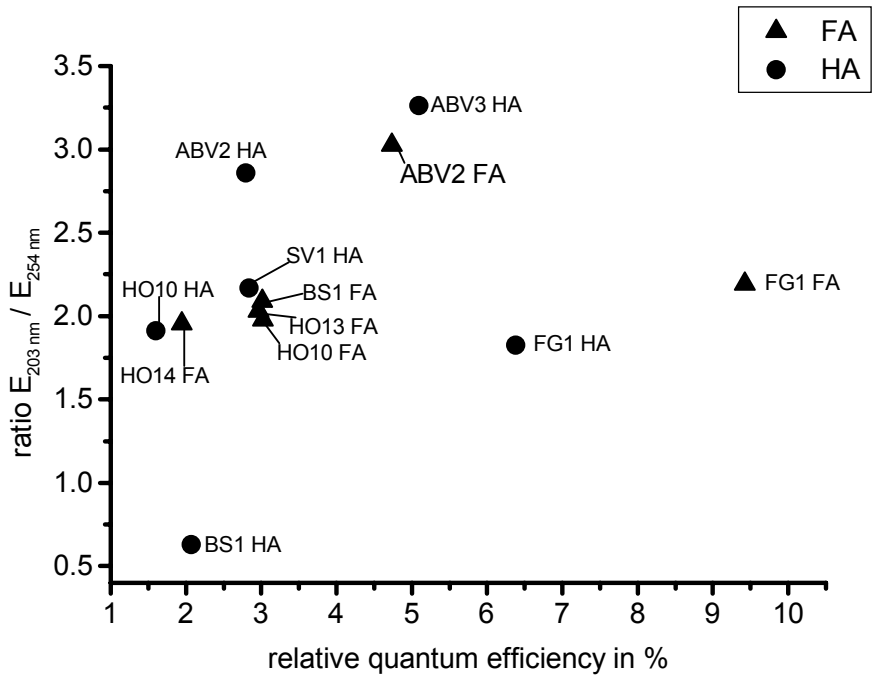

Figure 2.11.3-F4

Correlation of the RFQE (with λ_{ex} = 295 nm) with the ratio of the UV/Vis absorption at 254 nm and λ = 203 nm for FA fractions (triangles) and HA fractions (circles).

For the majority of HS investigated a reasonable correlation between the fluorescence maximum and ratio of Bz / ET bands is found, this holds especially for the FA fractions. The correlation with the RFQE was less successful when all data points are considered. However, the FG1 sample had originally a high iron content which made the sample very unstable and caused precipitation over time. Therefore, it is possible that the fraction of the HS which remained in solution had a relative higher fluorescence capability causing an overestimation of the RFQE. This could explain the observed large RFQE compared to the other FA and HA investigated.

2.11.3.2 Time-resolved fluorescence of HS

In addition to the steady-state fluorescence measurements time-resolved experiments were performed and the fluorescence decay of different HS was measured. These results are described in detail elsewhere and will be only summarized here (Kumke and Frimmel, 1996, Frimmel and Kumke 1998, Kumke et al. 1998a, Kumke et al. 1998b, Korshin et al. 1999). In general, for all samples a highly complex decay kinetic was found. Hence, sophisticated data analysis was absolutely required for an adequate data processing. The applicability of simplified approaches in which only a single decay time was used for the characterization of HS is very limited and a part of the information contained in the fluorescence decay data of HS is lost (Zimmermann et al. 1997, Illenseer 1999). Therefore, two different approaches were pursued in the decay data analysis. In the discrete component approach (DCA) a pre-set number of exponential decay terms was used. However, due to the heterogeneous character of HS the correct estimation of the pre-set number of decay components is crucial. Therefore, an alternative data analysis approach was used, in which no preassumed number of decay components was introduced. In this case, the fluorescence decays were evaluated with decay time distributions using the exponential series method (ESM) and the maximum entropy method (MEM).

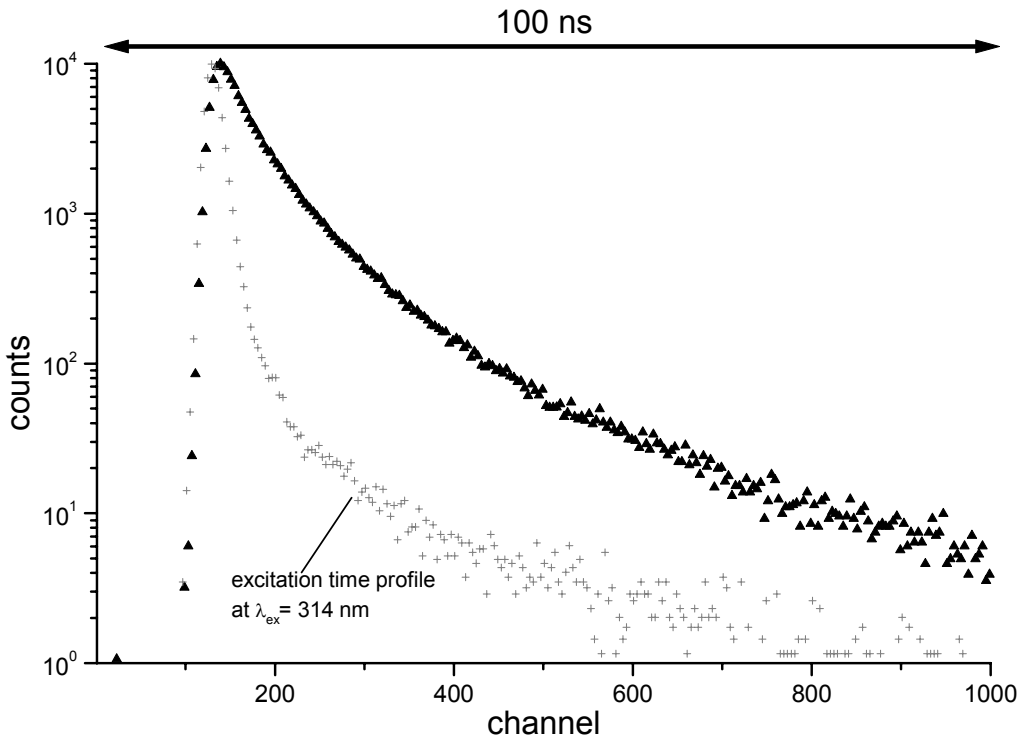

Figure 2.11.3-F5

Fluorescence decay of a soil seepage water (BS1 FA; λ_{ex} = 314 nm, λ_{em} = 450 nm).

In figure 2.11.3-F5 a typical fluorescence decay of HS investigated is shown. Two features are immediately obvious from figure 2.11.3-F5. First, the fluorescence decay is of higher order and second, the decay processes involved occur on a nanosecond time-scale (approx. < 50 ns).

In general, two main reason have to be considered to explain the observed complexity of the fluorescence decay of HS: i) HS are a complex, heterogeneous mixture of compounds and ii) various excited-state processes including conformational re-orientation as well as intra- and intermolecular proton transfer reactions. To account for the complexity in the DCA at least 3 exponential terms were necessary to obtain a reasonable data fit in terms of χ^2 and the randomness of the

residuals. In the DCA mean decay times in the range of τ_1 = 1 ns $\pm$ 0.5 ns, τ_2 = 4 ns $\pm$ 1 ns, and τ_3 = 10 ns $\pm$ 5 ns with the largest contribution of τ_2 were found for all HS investigated. It was already emphasized that the calculated decay times can not be readily ascribed to real chemical entities or substructures in the HS, and therefore, are operationally-defined (Kumke et al. 1998a). In the decay time distribution analysis the starting point was a flat distribution of 100 decay times (Kumke et al 1998a, Kumke et al. 1998b). The use of the distribution analysis was preferred since the heterogeneity of the sample could be better taken into account and thus, was less biased. It is interesting to note that in the decay time distribution analysis (ESM and MEM) a three-modal distribution of decay times was found as well. The mean decay times of each distribution peak were very similar to the decay times found in the DCA. In addition to decay times the width of the distribution peaks was obtained as well. It is tempting to relate the obtained peak width of the decay time distributions with the heterogeneity of the sample under investigation. For example, for some samples, (e.g., the waster water effluent ABV2 and ABV3 fractions), narrower peaks were found. It is tempting to assume for those samples a different stage of humification compared to the brown water or soil seepage water samples which are in an advanced humification stage.

The complexity of the fluorescence of HS is further reflected in the strong dependence of the fluorescence decay on λ_{ex} and λ_{em}. For the relative contribution of the mean decay time τ_2 = 4.3 ns values between 40 % and 75 % were determined in the wavelength range of 400 nm < λ_{em} < 520 nm for SV1 FA (Kumke et al. 1998b). On the other hand HO10 FA showed for τ_2 = 4.1 ns in the same emission wavelength range a much smaller variation.

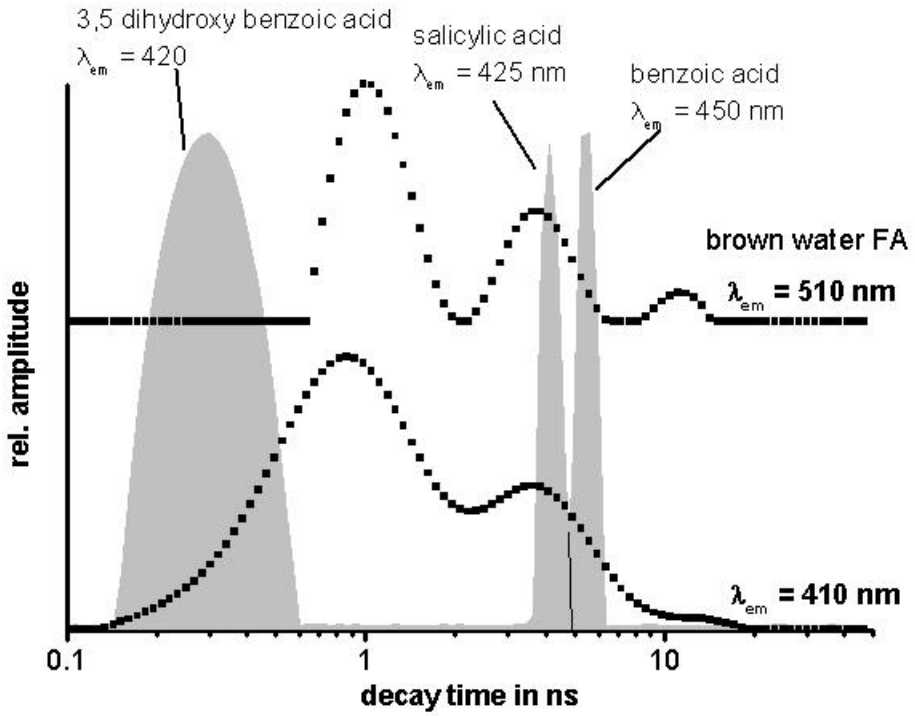

Figure 2.11.3-F6

Fluorescence decay distribution of a brown water FA (HO10 FA, λ_{ex} = 314 nm). Compared are the obtained fluorescence decay times of simple aromatic carboxylic acids. For all data evaluations the analysis was started with a flat distribution of 100 decay times in the time range between 0.1 ns < τ_i < 50 ns.

Individual compounds (e.g., salicylic acid), measured and analyzed under the same conditions, showed in distribution analyses sharp and mono modal distributions (Figure 2.11.3-F6). Using simple compounds as reference compounds, which are also assumed to be similar to precursors or building blocks for HS (e.g., aromatic carboxylic acid containing additional hydroxyl groups), a strong overlap with the second peak (around 4 ns) of the decay time distribution of HS was found (see figure 2.11.3-F6) (Frimmel and Kumke 1998).

2.11.3.3 Fluorescence of chemically-altered HS

Different HS altered by ozone, combined UV/H_2O_2, or by chlorination have also been investigated with spectroscopic methods (Korshin et al. 1998, Win et al. 2000). Under mild oxidation conditions (e.g., low ozone dose) an enhancement of the fluorescence intensity as well as a hypsochromic shift of the fluorescence maximum were observed. Furthermore, an overall shift from shorter to longer fluorescence decay times was observed in the distribution analysis of time-resolved fluorescence data. In combination with size-exclusion chromatography the results were discussed in terms of an apparent breakdown of larger HS molecules into smaller fragments (Korshin et al. 1998).

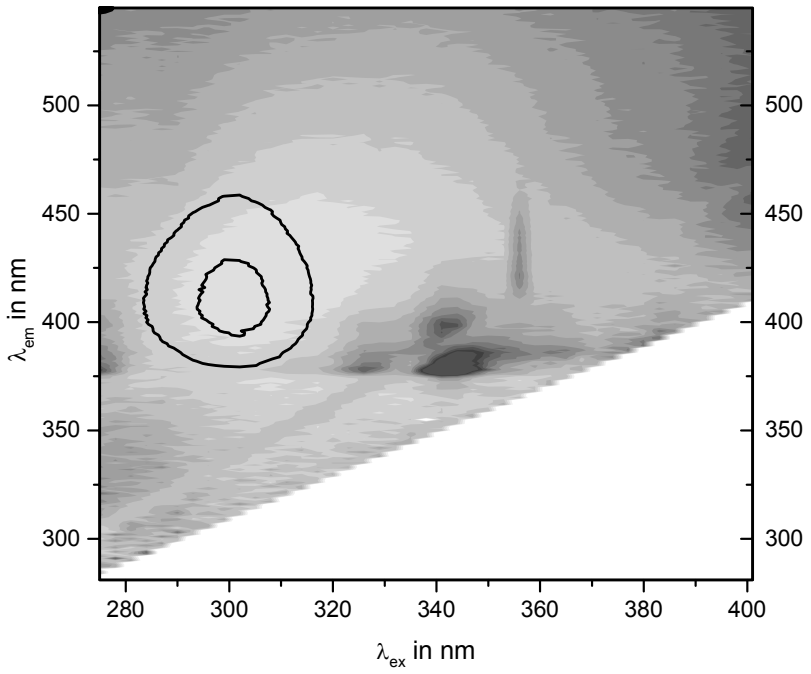

Figure 2.11.3-F7

Ratio of the total luminescence spectra of a brown water FA (HO10 FA) before and after chlorination (at a ratio of 5:10 of DOC : Cl). Compared is the TL spectrum of salicylic acid (black contour lines). Due to the chlorination a fluorescence increase was found (light area).

In figure 2.11.3-F7 the ratio of the TL spectra before and after chlorination of a brown water FA (HO10 FA) is shown. In the wavelength range marked by the light gray area (with a maximum around λ_{ex} = 300 nm and λ_{em} = 410 nm) a strong increase of the fluorescence intensity was observed since decreasing color corresponds to an increase in fluorescence enhancement. With an initial concentration of DOC of 5 mg/L the fluorescence intensity was increased by more than a factor of two. It is interesting to note that the wavelength range, in which the increase of the fluorescence intensity was observed, was almost independent of the oxidation process applied. Similar results were found in treatments of HS with low ozone doses (Win et al. 2000). In figure 2.11.3-F7 the spectral location of the TL spectrum of a

simple benzoic acid (salicylic acid) is shown as well (black lines). The emission maximum of salicylic acid falls well within the spectral range of the fluorescence increase caused by the oxidation treatment. For HS isolated from natural waters (e.g., bog water or soil seepage water) the observed fluorescence increase upon oxidation was strong while for HS isolated from a waste water effluent only a minor increase was observed. Very similar results were obtained for HS hydrolyzed under controlled experimental conditions. In case of HS, which was closely related to plant material precursors in the first place (e.g., brown water), a well pronounced fluorescence increase was observed in the same spectral regions of the TL spectrum. HS samples isolated from a waste water showed almost no change in the fluorescence properties upon hydrolysis. For those HS samples the UV/Vis spectrum stayed unchanged as well (Kumke et al. 2000c).

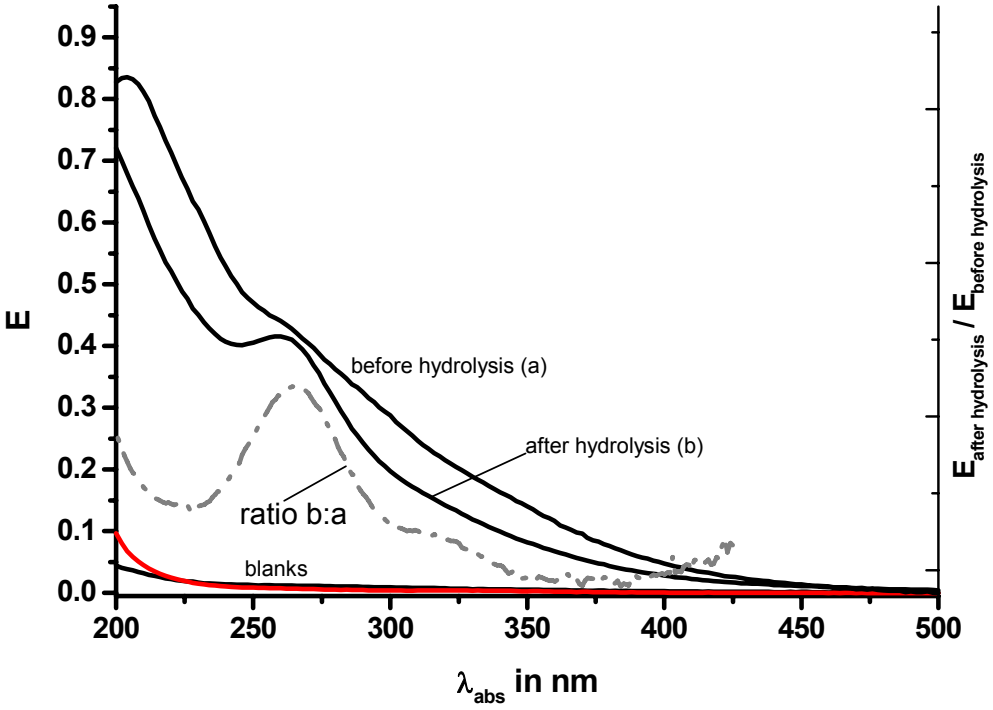

Figure 2.11.3-F8

UV/Vis absorbance spectra of a brown water FA (HO14 FA) before and after hydrolysis with 6 N NaOH at 100 °C. The DOC concentration was 5 mg/L. After the reaction the pH of the solution was adjusted to pH 7 using an ion exchange resin.

On the other hand, for samples with a possible strong contribution of plant material precursors (e.g., brown water HS, soil seepage HS), the unresolved absorbance spectrum was noticeably altered and a distinct peak appeared. In figure 2.11.3-F8 the absorbance spectra of a brown water FA before and after hydrolysis are shown together with the ratio of both spectra. Due to the hydrolysis new spectral features evolve from the unstructured background. The new absorption band at λ = 265 nm and a shoulder at λ = 315 nm and appear to be correlated with the observed increase in fluorescence intensity. This is indicated by the comparison of fluorescence

excitation spectra with a detection wavelength around 410 nm, which corresponds to the maximum of the observed fluorescence increase (Kumke et al. 2000c).

2.11.4 Conclusions

The simplest fluorescence approach used in HS studies is the measurement of standard 2D emission and excitation spectra. However, simple fluorescence intensity measurements of HS suffer from several limitations associated with the issues of comparability among different experimental conditions (concentration, pH, and chosen λ_{ex}) and the proper consideration of inner filter effects (Illenseer et al. 1999, Mobed et al. 1996). The latter limitation can be overcome without complex correction methods by using diluted solutions of HS. With the introduction of an external standard the dependence on experimental parameters can be minimized and the determination of a relative quantum efficiency becomes feasible. The usage of simples aromatic acids and their derivatives (e.g., salicylic acid, benzoic acid) appears justified because the substances can be considered as precursors in the generation processes or, alternatively, as degradation products in the breakdown of HS. It is therefore reasonable to assume these entities in HS as significant contributors in HS fluorescence and the use as reference fluorophores seems to be clearly justified. Compared to salicylic acid the fluorescence quantum efficiencies of the aquatic HS were found to be small (between 1 % and 5 % for the majority of HS investigated). Only for the FA and HA isolated from a ground water (FG1) higher values were obtained. There may be two main reasons for the low fluorescence intensities of HS. (1) Only a small number of chromophores, which are present in a reasonable high percentage since the color of HS is intense, are actively participating

in fluorescence, or (2) the fluorescence is reduced due to effective radiationless deactivation processes.

The fluorescence spectra were strongly dependent on the excitation wavelength. This supports the assumption that more than one type of fluorophore contributes to the emission. Depending on the excitation wavelength, at least two groups of fluorophores have to be considered. One group is excited in the UV region (Group I: 225 nm < λ_{ex} < 300 nm) and the second group (Group II) of fluorophores is excited at longer wavelengths. At excitation wavelength λ_{ex} < 300 nm both, Group I and Group II were excited. For Group I a relative weak fluorescence emission in the wavelength range 300 < λ_{em} < 370 was observed. Of course, within each group further heterogeneity due to slightly different chemical environments is present and this leads also to a spectral broadening. In the TL measurements the complete steady-state fluorescence characteristics of HS (of Group II fluorophores in the first place) was obtained. It was found that although the HS were isolated from different aquatic sources with quite different histories, the location of the emission and excitation maxima of the fluorescence spectra were very similar. This is especially valid for the FA fractions of the HS investigated which indicates that similar fluorophores are involved (at least for Group II). The width of the emission band should be indicative for the chemical (or environmental in terms of different functional groups) heterogeneity of the fluorescing sites. It is interesting to note that for the FA fractions very similar band widths were found while the HA fractions showed a larger variation in this parameter. It is tempting to attribute those effects to differences in molecular size and to a narrower distribution of fluorescing sites in smaller molecules. The observed Stokes shift between excitation maximum and emission maximum was huge. For all HS investigated, a Stokes shift > 100 nm was found (see figure 2.11.3-

F2). Again, for the FA fractions only a small variation was observed for this parameter. The large Stokes shift is a further indication that the fluorescence of HS is influenced (or determined) by the presence of intra- and intermolecular reactions in the excited state. An interpretation of the observed fluorescence of HS as only a sum of fluorophores is therefore definitely an oversimplification.

The strong involvement of intra- and intermolecular reactions in the fluorescence properties of HS is further supported by the results of the time-resolved fluorescence measurements. The experiments showed a highly complex fluorescence decay. The results obtained under variation of experimental conditions and after chemical modification of the HS support the interpretation in terms of intra- and intermolecular processes (e.g., proton transfer, conformational reorientation) (Frimmel and Kumke 1998, Kumke et al. 1998b, Illenseer et al. 1999).

The fluorescence intensity was significantly increased upon chemical reactions (e.g., oxidation, hydrolysis). In combination with size-exclusion experiments (SEC) is was shown that due to the chemical treatment the apparent molecular size was decreased. It is tempting to connect the observed increase in fluorescence intensity with a decrease in the structural disorder due to the reduction in molecular size. There are two aspects of structural disorder that have to be considered: disorder due to heterogeneity of compounds and disorder due to the flexibility of each HS molecule. Upon degradation into smaller fragments, the overall flexibility of the fluorophores is reduced and because of the presence of less vibrational and rotational degrees of freedom the fluorescence is increased. Furthermore, the introduction of hydroxyl and carboxyl groups in the aromatic fluorophores could lead to an increase as well. Recently, the absorption spectra of HS were discussed in terms of the Urbach theory (Mullins et al. 1992, Mullins and Zhu 1992, Illenseer et al.

1999). The results reported here add further evidence that HS have to be considered as highly structure-disordered systems.

2.11.5 Acknowledgements

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Authors

Dr. Michael Kumke

Institute of Physical Chemistry and Theoretical Chemistry, University of Potsdam

Karl-Liebknecht-Str. 24-25, 14476 Golm, Germany

Email address: Kumke@chem.uni-potsdam.de

Prof. F.H. Frimmel

Engler-Bunte-Institut, Division of Water Chemistry, University of Karlsruhe

Engler-Bunte-Ring 1, 76131 Karlsruhe, Germany

Email address: Fritz.Frimmel@ciw.uni-karlsruhe.de

Subject index

List of abbreviations and symbols

ABVx	waste water effluent sample (x = 2, 3)
BS1	soil seepage sample
Bz band	benzenoid band
χ^2	square of the error sum
DCA	discrete component approach
DOC	dissolved organic carbon
ESM	exponential series method
ET band	electron transfer band
FA	fulvic acid or fulvic acids
FG1	ground water sample
HA	humic acid or humic acids
HOx	brown water sample (x = 10, 13, 14)
HS	humic substances
λ_{ex}	excitation wavelength in nm
λ_{em}	emission wavelength in nm
$\lambda_{em,max}$	emission wavelength maximum in nm
MEM	maximum entropy method
MX	Z-2-chloro-3-dichloromethyl-4-oxo-butenoic acid
ν	wavelength in cm^{-1}
$\nu_{em,max}$	emission wavelength maximum in cm^{-1}
ns	nanosecond (10^{-9} s)
RFQE	relative fluorescence quantum efficiency
SEC	size-exclusion chromatography
SV1	brown coal production effluent sample

TL	total luminescence
τ	fluorescence decay time