

VARIATION OF THE UV-TO-BLUE FLUORESCENCE RATIO FOR ORGANIC MATTER IN WATER UNDER CONDITIONS OF FLUORESCENCE SATURATION

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ABSTRACT

Experimental results on non-linear fluorescence spectroscopy with laser excitation for organic matter in water are presented with emphasis on the variation of fluorescence spectral shapes through a wide range of the samples. Different types of organic material, such as algae cultures in water and aqueous solutions of commercially available DOM components (humic acid, fulvic acid, lignin, and mixtures of humic substances with phenolic compounds) were investigated. All substances under investigation show fluorescence saturation at both excitation wavelengths 266 and 355 nm used in the experiment. Excited at $I_{exc} = 266$ nm, (laser pulse duration $\tau_p = 0.3$ ns) *Chlorella* culture shows a different trend of the UV-to-blue fluorescence ratio compared to *Chlorococcum* culture and mixtures of humic substances and phenolic compounds with rising laser pulse power. Under certain conditions of excitation ($I_{exc} = 355$ nm, $t_p = 0.3$ ns) the fluorescence spectra of humic acid in water manifest inhomogeneous broadening. A «red shift» of the maximum position with increasing excitation photon flux is described for the first time. For a lignin solution in water no change in spectral shape with alteration of laser power was observed. We resume that samples of natural organic matter of different origin can be distinguished applying non-linear fluorimetry, even in the case of similar steady-state fluorescence spectra. Non-linear fluorimetry gives us also the possibility to verify hypotheses of the nature of fluorescence for complex organic substances.

INTRODUCTION

The compositional features of organic matter in water representing a dilute (~1 mg/L) but huge carbon reservoir (as big as atmospheric CO₂) offer clues with regard to sources and formation pathways of nonliving organic matter (1). Since the first reports on natural water fluorescence, spectroscopic methods have been widely applied for its characterisation. From the wide range of studies performed on natural waters of different origin it appears that organic matter may cause two distinct fluorescent components with emission maxima in the ranges 300...350 nm and 400...480 nm. The first component is attributed mainly to protein-like material (2-3). The contribution of other organic substances to this band has also been investigated (4). The second component has been assigned to humic substances or «gelbstoff» after Kalle (5).

For natural waters the position of the maximum for the gelbstoff fluorescence depends strongly on the excitation wavelength (6,7). This phenomenon illustrates the complexity of the fluorophoric structure of this material. It has been suggested that natural waters behave as a mixture of different groups of fluorophores (6). The gelbstoff fluorescence signal is dominated by the emission from fulvic acids that contribute up to 60 % of the total intensity (8). Continental and marine waters exhibit qualitatively similar featureless excitation and emission spectra; differences can only be noticed with precise and careful measurements of wavelength maxima and intensities (6). The fluorescence efficiencies defined as the ratio of fluorescence intensity versus

absorbance at excitation wavelength of humic, fulvic, and hydrophilic acids, which are representative of the main part of the fluorescent DOM, have been found to be different from each other (9). It was suggested to use fluorescence efficiency for a rough estimation of relative proportion of these DOM families and to characterise the evolution of the DOM while undergoing biological and photochemical transformations.

These findings stimulated the study of fluorescence techniques with laser excitation capable of characterising the origin of seawater DOM with remote sensing and of distinguishing it from organic pollutants (mineral oils, phenols, etc). One of such techniques can be non-linear fluorescence spectroscopy. Non-linear fluorimetry considers the case when pulsed laser sources are applied for spectra excitation and where the detected fluorescence intensity is not proportional to the excitation power (10-12). This deviation from linearity is typically noticeable under conditions of 10^{24} exciting photons per second and cm^2 , which can be easily achieved with lasers. Absorption cross-section, fluorescence lifetime, and other molecular characteristics rule the effect of fluorescence saturation. A comparison of the non-linear fluorescence response for the sample under investigation and model solutions with known dye characteristics can support or reject a hypothesis on the fluorophore nature.

This paper reports on fluorescence spectroscopy of organic matter with emphasis on variations of the spectral shape due to fluorescence saturation. A wide range of samples was analysed. Different types of organic material, such as DOM freshly produced and released by algae in water and aqueous solutions of commercially available DOM components were investigated. The fluorescence spectra are compared with those of natural seawater samples measured at different excitation wavelengths (13).

DESCRIPTION OF THE EXPERIMENT

Laboratory measurements of organic matter fluorescence emission were made at the LIDAR laboratory, ENEA, C.R.Frascati. The fourth or third harmonic of a Q-switched Nd:YAG-laser with 10 mJ pulse energy and about 0.3 ns pulse length were used for spectra excitation. Fluorescence emission was detected with an optical multichannel analyser (EG&G model OMA-III). Spectra were measured using an accumulation over 20 laser shots. Adjusting the delay time between the flashlamp trigger and the Q-switch of the laser allowed the pulse power to be attenuated over a wide range.

To avoid photobleaching of organic material caused by repeated laser pulses, the samples were analysed in a flow-through quartz cell of 1 cm path length with 266 nm excitation, or in a 5 litre container and constantly mixing the sample with 355 nm excitation. In this way each spectrum was acquired using a set of single-shot excited scans. However, even with these precautions fast photochemical reactions, which take place within the laser pulse duration, cannot be excluded. In this study they are not distinguished from fluorescence saturation effects caused by dynamic depletion of the molecular ground states.

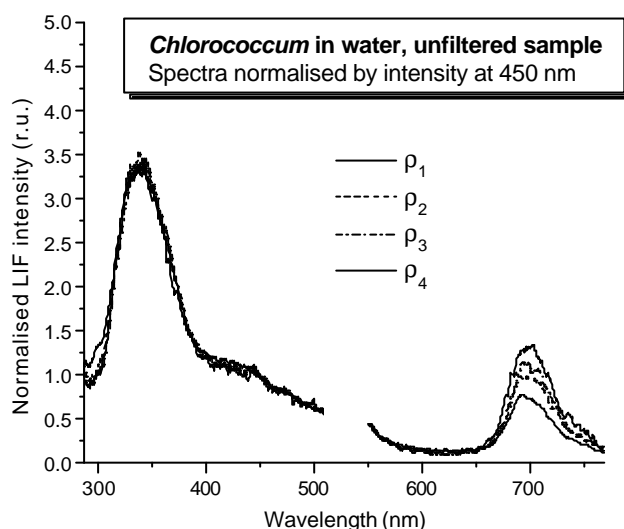
The algae cultures were grown at ENEA, C.R. Frascati, Italy. The samples of two freshwater algae cultures *Chlorella sorokiniana* and *Chlorococcum sp.* were taken at the stage of exponential growing. Concentration of chlorophyll *a* was estimated as 6,000 mg/ml for *Chlorella* and 1,600 mg/ml for *Chlorococcum*. Determination of chlorophyll (mg/m^3) concentration in algae and phytoplankton required measurements of absorbance by spectrophotometer at specified wavelengths and the use of these values in the trichromatic equations (14).

Aqueous solutions of DOM components were prepared in Milli-Q water from commercially available chemicals: liginosulfonic acid (Aldrich), humic acid (Aldrich), pyrogallol (Fluka), and gallic acid (Fluka). The fulvic acid employed for fluorescent measurements was the Suwannee River fulvic acid standard reference material of the International Humic Substance Society.

LASER- INDUCED FLUORESCENCE OF ALGAE CULTURES

Laser induced fluorescence spectra of algae cultures in water are shown in Figures 1-2 for several values of the excitation photon flux. The fluorescence spectra have two emission peaks at 325 and 440 nm attributed to protein-like and gelbstoff-like fluorescence typically observed for natural water excited at 266 nm. The fluorescence intensity of both bands is a non-linear function of the excitation laser pulse power. The initial flux corresponding to non-attenuated laser pulses was equal to $\rho_1 \sim 10^{25} \text{ cm}^{-2}\text{s}^{-1}$. The laser beam intensity was attenuated by factors of two (ρ_2), five (ρ_3) and 20 (ρ_4).

To compare their band shape, the spectra are normalised by the intensity of fluorescence emission at 450 nm. It is clearly seen for the *Chlorococcum* culture in water, that the UV-to-DOM fluorescence ratio keeps constant for all photon fluxes used, while the Chlorophyll-to-DOM fluorescence intensities ratio



increases remarkably along with the attenuation of the laser pulse power (Figure 1).

Figure 1: Laser-induced fluorescence spectra of a *Chlorococcum* culture in water measured under conditions of fluorescence saturation.

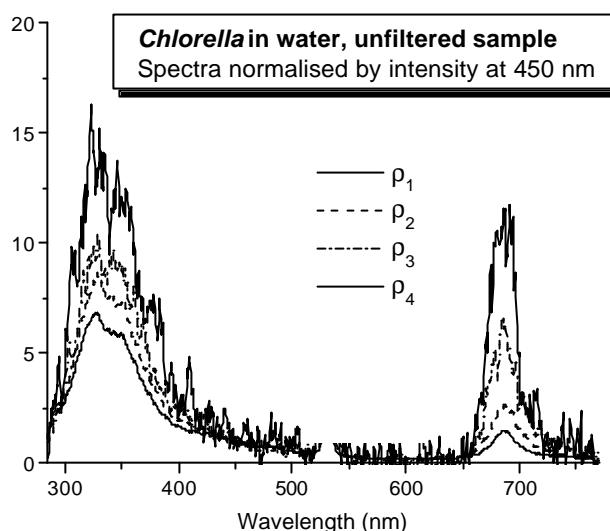


Figure 2: Laser-induced fluorescence spectra of a *Chlorella* culture in water measured under conditions of fluorescence saturation.

For the normalised spectra of the *Chlorella* culture in water (see Figure 2) the UV-to-DOM fluorescence ratio decreases twice with attenuation of the initial laser power 20 times. The Chlorophyll-to-DOM fluorescence intensities ratio decreases more than five times along with the equal attenuation of the laser pulse power.

A similar behaviour of the fluorescence spectra for both algae cultures was found also for the filtered samples. They do not show chlorophyll fluorescence, and the ratio of UV-to-blue fluorescence keeps constant for the filtrate of the *Chlorococcum* sample and decreases 1.5 times for that of the *Chlorella* sample with rising laser pulse power.

In both emission bands there is no shift in the position of the fluorescence maximum for all the algal samples under investigation – both filtered and unfiltered.

LASER-INDUCED FLUORESCENCE OF MIXTURES OF HUMIC SUBSTANCES AND PHENOLS

Two solutions were prepared by mixing solutions of humic substance (humic acid or fulvic acid) and of phenolic compounds (gallic acid or pyrogallol). Gallic and pyrogallol are regarded as products of DOM decay in a natural reservoir and may represent structural links of natural polymers. Their concentration were 2.5 mg/l of pyrogallol and 5 mg/l of fulvic acid for the first mixture, and 0.2 mg/l of gallic acid and 2 mg/l of humic acid for the second mixture.

Figure 3 shows laser-induced fluorescence spectra of the second mixture for different values of excitation pulse power. The initial flux corresponding to non-attenuated laser pulses was equal to $\rho_1 \sim 10^{25} \text{ cm}^{-2}\text{s}^{-1}$. The laser beam intensity was attenuated 1.5 (ρ_2), 2 (ρ_3) and 5 (ρ_4) times. The spectra are normalised to the intensity of the fluorescence response at 450 nm to compare their shapes.

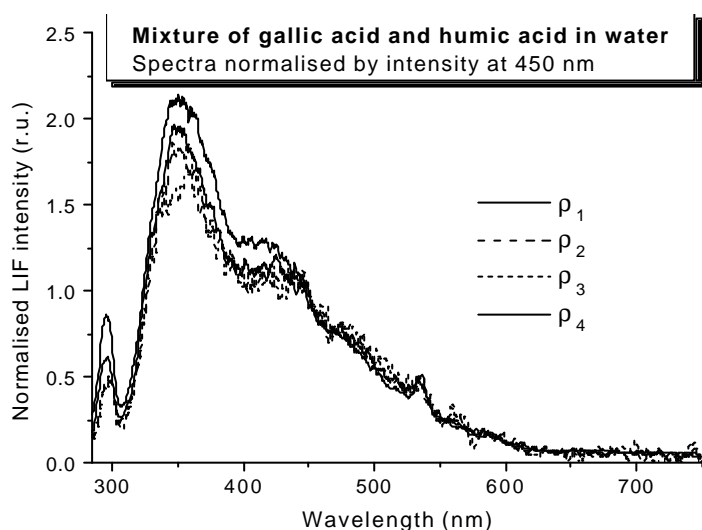


Figure 3: Laser-induced fluorescence spectra of a mixture of gallic acid and humic acid in water measured under conditions of fluorescence saturation.

Fluorescence spectra of both mixtures have overlapping emission bands. The UV fluorescence with maxima at 350 nm corresponds to phenolic substances (4), while humic substances emit in the visible spectral region. For both emission bands the normalised fluorescence intensity was found to be non-linear with the excitation intensity due to fluorescence saturation effects.

As shown in Figure 3, the ratio of UV-to-blue fluorescence increases for the solution with rising laser pulse power. The same behaviour was observed for the mixture of pyrogallol and fulvic acid. In contrast to fluorescence spectra of DOM in algae samples the ratio of UV-to-blue fluorescence increases for solutions with rising laser pulse power.

INHOMOGENEOUS SPECTRAL BROADENING OF HUMIC COMPOUNDS

Figure 4 shows fluorescence spectra of humic acid (concentration 0.1 mg/l) and lignin (concentration 4 mg/l) in water excited at 355 nm at different degrees of laser pulse attenuation. For humic acid in water the non-attenuated initial excitation photon flux was $\rho_1 \sim 8 \cdot 10^{24} \text{ cm}^{-2}\text{s}^{-1}$ ($\rho_3; \rho_2; \rho_1 = 4:20:80$), and for lignin solution $\rho_1 \sim 10^{25} \text{ cm}^{-2}\text{s}^{-1}$ ($\rho_3; \rho_2; \rho_1 = 0.4:60:100$). Both substances show a strong saturation with increasing laser pulse power. The normalised fluorescence decreases by a factor of three for humic acid and for lignin under comparable conditions of saturation from $5 \cdot 10^{23} \text{ cm}^{-2}\text{s}^{-1}$ to the non-attenuated pulse power.

The fluorescence spectra of humic acid and lignin shown in Figure 5 are normalised to the maximum fluorescence to compare the shapes of the spectra excited at different degrees of laser pulse attenuation. It is clearly seen that both the maximum position and bandwidth of humic acid fluorescence are dependent on

the excitation intensity. A shift towards longer wavelengths, or a «red shift», up to 30 nm was found with increasing laser pulse power. No change in spectral shape was observed for the lignin solution with alteration of the laser power by two orders of magnitude (Figure 5).

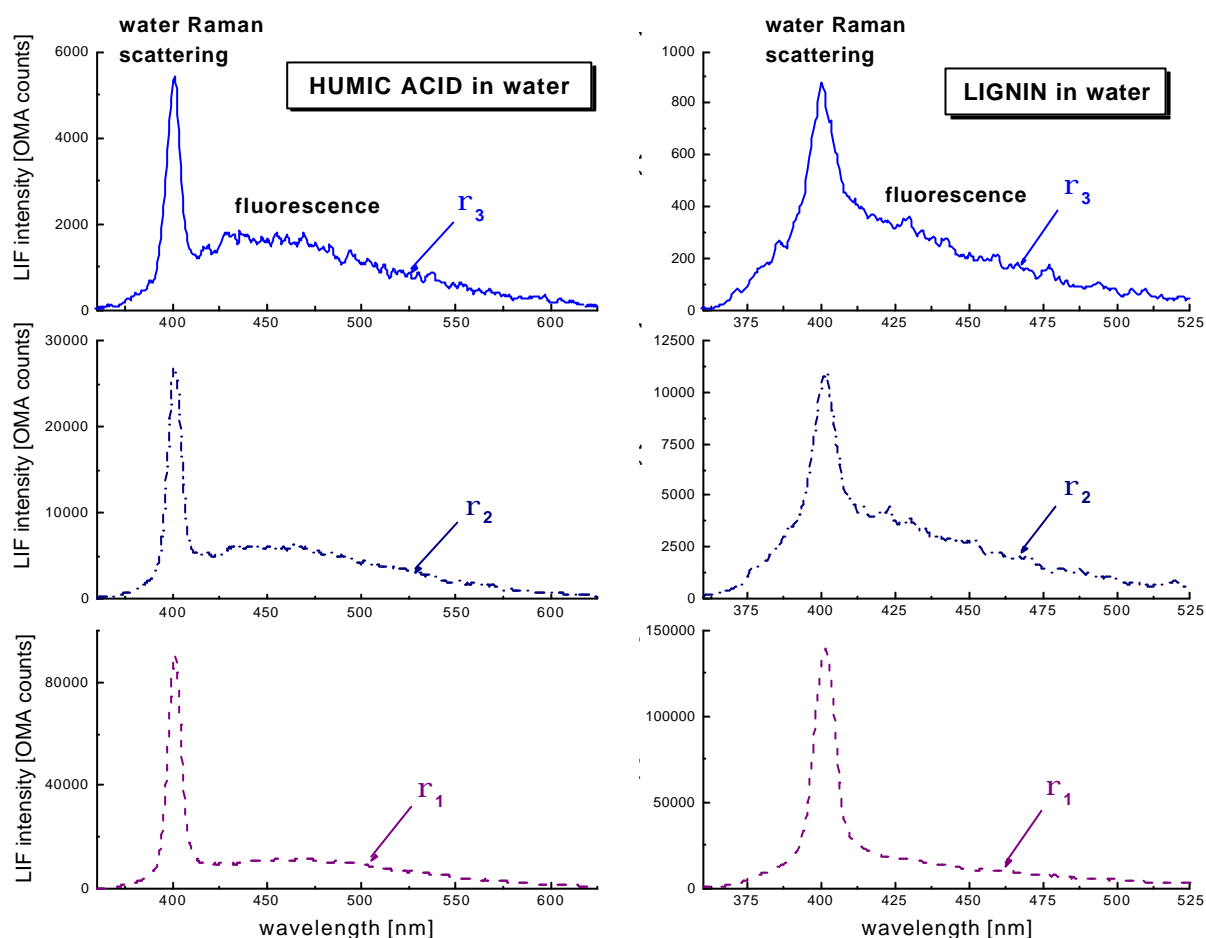


Figure 4: Fluorescence saturation for humic acid (left) and lignin (right) in water.

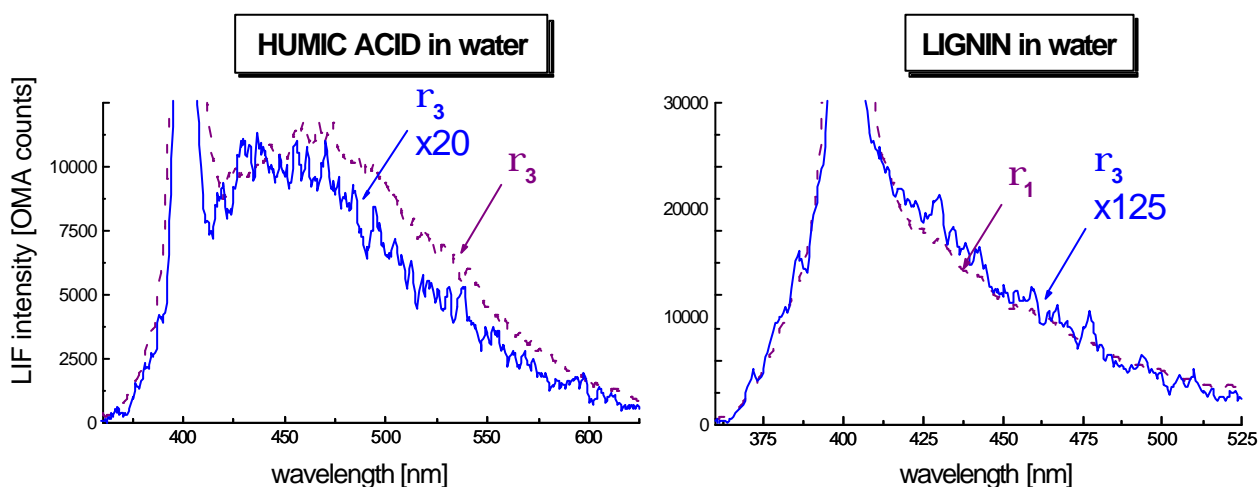


Figure 5: Fluorescence spectra of humic acid (left) and lignin (right) in water normalised to the fluorescence maximum.

DISCUSSION OF EXPERIMENTAL RESULTS

For all investigated samples an effect of fluorescence saturation was found which appears as a non-linear fluorescence response versus excitation laser power. This leads to a variation of up to a factor of five of the fluorescence intensity normalised to the water Raman scattering signal. As it is known from the theory of fluorescence saturation (12), mixtures of distinct fluorophores have different degrees of fluorescence saturation, and hence the ratio of their fluorescence amplitude varies with the laser pulse power. In accordance with these findings we expected to observe an alteration of UV-to-blue fluorescence ratio for samples containing distinct fluorophores.

The UV-to-blue fluorescence ratio changes with rising laser pulse power for some of the samples – mixtures of phenolic compounds and humic substances, samples of *Chlorella*, but keeps constant for other samples - seawater (13), unfiltered sample of *Chlorococcum*. Results on seawater photobleaching (7,13) and data obtained with sample filtration support the different chemical natures of chromophores responsible for the UV and the visible emission in algae cultures and natural water samples. However, for seawater and *Chlorococcum* culture strong laser pulses saturate both the UV and the visible fluorescence bands to the same extent. The ratio of two fluorescence amplitudes keeps remarkably constant, while the laser pulses are attenuated by two orders of magnitude. This finding can be interpreted as evidence that both fluorophores responsible for the UV and the visible emission are bound within the macromolecule, and the intramolecular energy transfer processes from absorbing centres to fluorophores take place.

We did not observe changes in the maxima position both for the UV and visible fluorescence for all samples with 266 nm excitation. In contrast, the fluorescence excited at 355 nm demonstrated inhomogeneous spectral broadening of gelbstoff fluorescence for two samples. A remarkable «red shift» of the maximum position in 30 nm was observed for a humic acid solution with increasing excitation photon flux. The «red shift» apparently was not caused by photodegradation of organic material in water by laser pulsed irradiation, since it was measured in the 5-litre container with a constantly mixed sample. A weak «red shift» of maximum position in 5 nm was observed for the sample of seawater excited at 355 nm under conditions of fluorescence saturation (13). The «red shift» of maximum position for humic substances can be explained in terms of direct excitation at 355 nm of various fluorophores contributing to the gelbstoff fluorescence emission. The results of previous studies (2, 6-9) confirm that DOM in natural waters behaves as a mixture of different groups of fluorophores. Direct excitation at 355 nm of individual fluorophores leads to selective saturation of their fluorescence and changes their contribution to the integral DOM fluorescence. The excitation at 266 nm corresponds to indirect excitation of fluorophores emitting in the visible range, so there is no noticeable shift of the maximum position for different samples, including above mentioned solution of humic acid in water.

Other complex organic compounds have a non-linear fluorescence response under excitation at 355 nm different from that for humic acid. For lignin solution in water under conditions of fluorescence saturation no change was observed in the spectral shape with alteration of laser power ($\lambda_{\text{exc}} = 355 \text{ nm}$) by a factor of 250. For several crude oils the «blue shift» of maximum position was observed (15) under the same condition of excitation as for humic acid.

The main results of non-linear fluorescence spectroscopy, which are important for DOM remote sensing, are summarised in Table 1. We resume that water samples can be classified into different types applying non-linear fluorescence spectroscopy with laser excitation. The characteristics of interest are the UV-to-blue ratio of emission intensities excited at 266 nm, and the manifestation of a spectral shift upon variable laser-pulsed excitation.

CONCLUSIONS

This paper is devoted to fluorescence spectroscopy of organic substances in water with emphasis on variations of the spectral shape due to fluorescence saturation through a wide range of the samples. The main conclusions can be formulated as follows:

- All water samples under investigation demonstrate a non-linear response of the fluorescence intensity versus excitation power, i.e. the effect of fluorescence saturation at both excitation wavelengths 266 and 355 nm.
- Excitation at 266 nm shows different trends of the UV-to-blue fluorescence ratio with rising laser excitation power for *Chlorella* culture, *Chlorococcum* culture in water and mixtures of phenolic compounds with humic substances. For the solutions of chemicals with two distinct fluorophores the ratio increases; for *Chlorococcum* culture and seawater sample it keeps constant; for *Chlorella* samples the ratio decreases.
- Under certain conditions of excitation ($I_{exc} = 355$ nm, $t_p = 0.3$ ns) the fluorescence spectra of humic acid in water manifest inhomogeneous broadening, which leads to a «red shift» of the maximum position with increasing excitation photon flux. A lignin solution in water did not change its spectral shape with alteration of laser power, for crude oils the «blue shift» of the maximum position was found.
- Water samples can be classified into different types applying non-linear fluorescence spectroscopy with laser excitation. The characteristics of interest are the UV-to-blue ratio of emission intensities and the manifestation of a spectral shift upon variations of the laser-pulsed excitation. Non-linear fluorescence spectroscopy enables us also to verify hypotheses of the nature of fluorescence for complex organic substances.

Table 1: Changes in fluorescence spectra due to increasing laser pulse power.

| 266 nm excitation | Sample | UV-to-blue fluorescence ratio |
|-------------------|---|----------------------------------|
| | unfiltered natural water | keeps constant or decreases |
| | Samples of <i>Chlorella</i> | keeps constant or decreases |
| | Samples of <i>Chlorococcum</i> | keeps constant |
| | model solutions of humic substances with phenolic compounds | Increases |
| 355 nm excitation | Sample | Position of fluorescence maximum |
| | seawater DOM | very small «red shift» |
| | humic acid in water | «red shift» |
| | lignin in water | keeps constant |
| | crude mineral oils | «blue shift» |

ACKNOWLEDGEMENTS

This work was supported by the fellowship of the University of Bordeaux and the ENEA fellowship.

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