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Standardization of fluorescence excitation–emission-matrices in aquatic milieu

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ABSTRACT

Fluorescence excitation–emission-matrices (EEM) are a useful tool for water quality monitoring. Recent publications show the potential of the method for real time drinking water control. However, in fluorescence measurements there is still a need for standardization to make data interpretation comparable. In this work a standardization procedure based on excitation and emission correction as well as normalization and optional inner filter effect correction is presented. By measurements of humic acid and tryptophan standards with two different spectrometers (LS 50 and LS 55 by PerkinElmer) the procedure application leads to comparable fluorescence intensities with relative standard deviations (median) of 6.6–8.4% and 10.6–12.0%, respectively. These small differences are not avoidable even if all possible correction methods are implemented and constant measurement conditions are given. The used BAM kit for emission correction induced good agreement in peak shape not only for single wavelengths but also for the whole EEM. As a consequence it is necessary to use identical equipment and identical experimental conditions in order to apply this method in fields of water quality control if small changes of fluorescence intensities are relevant for data assessment.

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1. Introduction

Fluorescence spectroscopy is a widely used method for analysing water samples of different origin. It is highly sensitive and selective and can give a wide spectrum of information in very short time. Measurements result in an excitation–emission-matrix (EEM) in which the substances of dissolved organic matter (DOM) appear in specific areas. Since an EEM works like a finger print researchers use this to identify the origin of analysed water [1–4]. In former years the method was used for sea water and river water monitoring [5–7]. A comparatively new field of application is controlling tap water and drinking water treatment trains [8]. The interest of applying this spectral method in fields of water technology is increasing because of its sensitivity and real time character.

At the AGU Chapman Conference on Organic Matter Fluorescence 2008 in Birmingham (UK) the state of scientific and technical knowledge was discussed. Though it is possible to give information about the composition and characteristics of DOM and also about its origin and distribution, there is still a great need for research on how to quantify fluorescence signals. In this context there are two main problems: the first one is the comparability of results of measurements achieved by different instruments and the second one is quantification of signals affected by fluorescence quenching effects.

In respect of comparability of results from different working groups in this field worldwide, it is necessary to create a uniform standardization procedure. Every fluorescence spectrometer gives only device-dependent signals. This is due to different light-sources, different models of photomultipliers with different wavelength sensitivity. Even between devices of the same model and manufacturer it is difficult to get similar signals, because of the aging impact of device components [9,10]. Therefore measurements have to be corrected by a standardization procedure which includes an excitation and an emission correction step as well as a normalization step. The aim of standardization is making measurements independent from the used spectrometer device and its aging effects.

Concerning quantification of signals there is still the problem of fluorescence quenching. The term quenching includes so called 'Inner Filter Effects' (IFE), caused by the absorption of light by the sample, and the collision or complex formation between a fluorophore and another molecule. Both result in a decrease of fluorescence intensity making it more difficult to quantify a signal.

The aim of this work is to present a standardization procedure following Lawaetz and Stedmon [9] and Murphy et al. [11]. In contrast to Lawaetz and Stedmon, in this work the focus lays on a comparison of the standardization of a whole EEM. This is of high interest because the whole matrix gives a better impression

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of the pollution level of water and it characterizes relevant components of total organic carbon (TOC). Moreover it is pointed out that using the emission correction kit of the Federal Institute for Material Research and Testing (BAM, Germany) is appropriate to eliminate differences in spectral shape in emission mode.

Finally the order of existing standardization procedures is modified by setting inner filter effect correction as last step.

2. Procedures

2.1. Standardization procedure

Standardization of data is important for comparing results of different measurements. In spectrometers several components are responsible for artefacts. These are created for example by excitation as well as emission monochromator, lamp or photomultiplier. Temperature is also decisive for fluorescence intensity. Without any regulation during the measurement temperature is not exactly on the same level in different devices. All of these influences can deform and dislocate peaks in a spectrum. Therefore measurements have to be corrected with respect to excitation and emission. Experimental conditions are supposed to be non-varying.

For excitation correction in some devices the light signal is split before reaching the sample and a part of the light is measured by a reference photomultiplier. The sample signal is divided by the reference photomultiplier signal (SRPM) and by the so-called correction spectrum (C_{ex}), as it is done by the PerkinElmer devices used for this work. An automated excitation correction is established in many systems and therefore governed by the manufacturers. The correction spectrum is measured during the fabrication process and saved on the hardware of the system. In some cases it could be necessary to create an own excitation correction spectrum or function. This could be done by a quantum counter as Rhodamine 101 [12].

The emission correction spectrum in contrast has to be established by the user. A correction spectrum relates every emission wavelength to a correction factor whereby the sample signal is multiplied with and therefore corrected (C_{em}). Such a spectrum can be created by using fluorescence dyes and their reference spectra. The Federal Institute for Material Research and Testing (BAM, Germany) and the National Institute of Standards and Technology (NIST, Gaithersburg, USA) have created useful correction tools during the last years [9,10], from which the BAM kit is used in this work.

By doing an excitation and emission correction all device dependent aspects, which affect the spectral position and shape of a fluorophore, can be removed. The last step in standardization is the normalization of intensities. There are different possibilities for unification, i.e. using quinine sulfate-equivalents [13,14] or Raman normalization which is most commonly used although it is done in many different ways. Some researchers use the intensity of the Raman scatter peak at a certain excitation–emission-wavelength combination [15]. However, Lawaetz and Stedmon [9] show that it is more robust to take the area of the Raman peak at a certain excitation wavelength as normalization factor.

For normalization the approach of Lawaetz and Stedmon is used, which consists of calculating the area under the Raman peak of pure water at λ_{ex} = 350 nm and 371 nm $\leq \lambda_{em} \leq$ 428 nm. This spectral range should be appropriate for devices with UV sensitive as well as red sensitive photomultipliers. The Raman peak should be measured of pure water, sealed in a standard one cm cell (e.g.: PerkinElmer water cuvette). The advantage of using a sealed standard water cell is the high robustness and reproducibility of the peak. Moreover there is not any risk of having dilution errors, like in quinine sulfate dilution series for normalization.

For each excitation–emission wavelength in an EEM the standardization procedure can be mathematically described by the following equations

$$F_{stand}(\lambda_{ex}, \lambda_{em}) = F_{obs}(\lambda_{ex}, \lambda_{em}) \cdot C_{ex}(\lambda_{ex}) \cdot C_{em}(\lambda_{em}) \cdot n[\text{r.u.}] \quad (1)$$

$$n = \frac{1}{\text{Area}_{ram}} \quad (2)$$

$$\text{Area}_{ram} = \int_{371\ \text{nm}}^{428\ \text{nm}} F_{obs}(\lambda_{ex} = 350\,\text{nm}, \lambda_{em}) \cdot C_{ex}(\lambda_{ex} = 350\,\text{nm}) \cdot C_{em}(\lambda_{em}) d\lambda_{em} \quad (3)$$

F_{stand} is the standardized fluorescence intensity at a certain excitation (λ_{ex}) and emission (λ_{em}) wavelength. F_{obs} is the observed raw intensity from the spectrometer device, C_{ex} the excitation correction spectrum, C_{em} the emission correction spectrum and n is the normalization factor. It is important to note that the spectrum of the Raman peak of the sealed standard water cell is excitation and emission corrected, too. The standardized intensity has the unit r.u. (Raman unit). With normalization by Raman peak the measurements become independent of the chosen multiplier voltage.

After this standardization procedure a correction for inner filter effects can be done by the following equation, if needed.

$$F_{IFE}(\lambda_{ex}, \lambda_{em}) = F_{stand}(\lambda_{ex}, \lambda_{em}) \cdot C_{IFE}(\lambda_{ex}, \lambda_{em}) \quad (4)$$

F_{IFE} is the standardized and IFE corrected fluorescence intensity and C_{IFE} the IFE correction factor, calculated by a method of user's choice (Lakowicz [12], Gauthier et al. [16], Larrson et al. [17]).

This approach of a final IFE correction differs from other approaches as suggested by Lawaetz and Stedmon [9] or Murphy et al. [11]. It is important to notice that standardization and correction of IFE are completely different aspects. Standardization aims at eliminating device dependent factors, which distort both the excitation shape and emission shape of a fluorophore. By standardization device independent matrices are created, scaled to the physically universal scatter light of pure water. In contrast to that inner filter effects are independent from spectrometer devices. The level of IFE depends only on absorption of the sample itself. Another reason for treating IFE correction independently from standardization is the fact that sample conditions determine the necessary IFE correction procedure, which hence needs to be chosen freely. Well known procedures are corrections by Lakowicz [12], Gauthier et al. [16] and Larsson et al. [17]. The methods of Lakowicz and Gauthier are based on an additional absorption spectrum of the sample, measured by an additional absorption spectrometer. Larsson's approach is based on the Raman scatter peak of the sample and does not need an extra measurement. Each method has its own advantages and disadvantages. It is the researcher's duty to choose the appropriate approach in each particular case. For example, using cut-off filters to remove scatter light in the sample makes it impossible to apply Larsson's method. On the other hand using the corrections of Lakowicz or Gauthier without having an absorption spectrometer is not possible either. Moreover, every absorption spectrometer has different device specifications, too. Measuring the absorbance with different slit widths, like 0.5 nm or 2 nm will finally lead to different results after IFE correction. Comparing two fluorescence spectra, standardized by an approach which includes IFE correction in the procedure, can finally lead to different results, because of differences in the absorbance spectrometer devices although the excitation and emission correction and normalization steps produced similar spectra. Besides, there is another reason, which allows changing the standardization order. Looking at Eqs. (1) and (4) the complete procedure of standardization and IFE correction is

simply a multiplication of raw fluorescence intensity with several factors, which can be changed in their order as needed.

2.2. Inner filter effect correction methods

Inner filter effects need to be corrected since they deplete the fluorescence signal affecting the desired linear relationship between concentration of fluorophore and fluorescence intensity. There are primary and secondary inner filter effects. Primary inner filter effects originate in absorbance of light with excitation wavelength. Secondary inner filter effects on the other hand originate in absorbance of light with the emission wavelength of the fluorophore. For best results both effects should be included in a correction procedure.

Whether inner filter effects have to be considered or not should be tested by UV/VIS-measurements. From an absorbance of 0.01–0.06 or greater inner filter effects appear. Correction is possible until an absorbance of 2 [18], above this irreversible loss of fluorescence signal is caused.

The most frequently applied correction procedures are explained below.

2.2.1. Gauthier (GAU)

The correction by Gauthier is done using

$$F_{\mathrm{corr}} = F_{\mathrm{obs}} \frac{2.3 d O_{\mathrm{ex}}}{1 - 10^{-d O_{\mathrm{ex}}}} 10^{d_2 O_{\mathrm{em}}} \frac{2.3 w_2 O_{\mathrm{em}}}{1 - 10^{-w_2 O_{\mathrm{em}}}}, \quad (5)$$

where F_{corr} is the corrected fluorescence intensity, F_{obs} the measured intensity which can be replaced by F_{stand}, O_{ex} is the absorbance at excitation wavelength, O_{em} the absorbance at emission wavelength, d is the thickness of the cuvette, w_2 the inner width of the cuvette and d_2 the thickness of the cuvette wall both measured in the direction of the emission wavelength [16]. By using the absorbance at emission wavelength secondary inner filter effects are corrected additionally to the primary inner-filter effects.

2.2.2. Lakowicz (LAK)

The correction method of Lakowicz uses

$$F_{\mathrm{corr}} = F_{\mathrm{obs}} \times 10^{[(O_{\mathrm{em}} + O_{\mathrm{ex}})/2]}. \quad (6)$$

This correction procedure is a simplification of the correction of Gauthier. The path of the exciting light is assumed to be equal to the path of the emitted light [12]. Primary inner-filter effects are corrected as well as secondary inner-filter effects.

2.2.3. Larsson (LAR)

The approach of the correction of Larsson is different from all of the above mentioned. It uses the Raman peak of the sample which is influenced by the number of absorbing molecules [17]. A correction is done by using

$$F_{\mathrm{corr}} = F_{\mathrm{obs}}(\lambda_{\mathrm{ex}}) \frac{r_{\mathrm{blind}}(\lambda_{\mathrm{ex}})}{r_{\mathrm{obs}}(\lambda_{\mathrm{ex}})} \quad (7)$$

whereas $r_{\mathrm{blind}}(\lambda_{\mathrm{ex}})$ is a vector which stores the area of the Raman peak at every measured excitation wavelength of the blank sample. The vector $r_{\mathrm{obs}}(\lambda_{\mathrm{ex}})$ stores the area of the Raman peak at every excitation wavelength of the sample. Integration is done using the trapezium formula.

3. Experimental methods

For this work tryptophan ((S)-(−)-tryptophan, Merck 816017) in a concentration of 20 μg/L and 100 μg/L in Milli-Q-water (pH 5.76 and 5.8) and a humic acid (Humic Acid Suwannee River Standard II from the IHSS) in a concentration of 2 mg/L and 10 mg/L (pH 5.5 and 5.2) were used. The choice of concentration levels is orientated at typically appearing concentrations in natural waters.

Fluorescence was measured with a LS 50 and a LS 55 (PerkinElmer) spectrometer. The slit widths were set to 10 nm in excitation and emission monochromators. For tryptophan the excitation wavelength was varied between 220 nm and 300 nm, with an increment of 10 nm. The emission wavelength was varied between 300 nm and 500 nm with 0.5 nm increment. For the humic acid standard a window of λ_{ex} = 220–440 nm and λ_{em} = 350–600 nm was used, with the same λ_{ex} and λ_{em} increments as used for tryptophan. To eliminate scatter peaks a 290 nm emission cut-off filter was used for tryptophan and a 350 nm cut-off filter for humic acid.

The scan speed was set to 1200 nm/min. The temperature was adjusted to 25 °C using the electronic Peltier thermostat PT 31 (A. Krüss Optronic, Germany).

Cuvettes for absorption and fluorescence measurements had a base area of 1 cm^2. The thickness of the walls was assumed to be negligible.

3.1. Standardization

Excitation correction was done by using the correction spectrum stored in the instruments. For emission correction a correction function was calculated using the spectral fluorescence correction kit of BAM (Fluka, 97003). Data was normalized to the integral of the Raman peak of a standard water cuvette (PerkinElmer, L2251293) at 350 nm excitation and 371–428 nm emission wavelengths. Before and after each measurement the Raman peak was measured. For standardization the average of both peaks was used.

Each solution was measured 3 times on each device. The same was done for a Milli-Q blank. In order to compare the spectra between both instruments the relative standard deviation, S_{rel},

$$S_{\mathrm{rel}} = \sqrt{\frac{\sum_{i-1}^{n} (x_i - \bar{x})^2}{n - 1}}, \quad (8)$$

was calculated out of six measurements (3 at LS 50 and 3 at LS 55), where n is the number of measurements and x_i is the ith measurement.

3.2. Inner filter effect correction

For inner filter effect correction the Humic Acid Suwannee River Standard II from the IHSS was used. Twelve concentrations between 1 and 30 mg/L were measured, 1–10 mg/L (in 1 mg/L steps), 20 mg/L and 30 mg/L.

Absorption spectra were measured with Lambda 25 (PerkinElmer). The slit width was set to 1 nm and scanning speed was set to 480 nm/min.

For validation of the different inner filter effect correction methods a linear regression of the corrected samples has been done and the R^2 values were calculated by

$$R^2 = 1 - \ldots \frac{\sum (\hat{y}_i - y_i)^2}{\sum (y_i - \bar{y})^2} \quad (9)$$

whereas i represents the concentration, n represents the maximum of the measured concentrations, $\hat{y}$ is the value calculated by the regression line, y the corrected value and $\bar{y}$ the average of the corrected values. In this context R^2 evaluates the linearity of the inner filter effect corrected values.

3.3. Blank subtraction

Blank subtraction was done either after standardization or after performing IFE correction. Neglecting IFE correction, the subtraction has to be done after standardization of both sample and blank

Table 1
Median and mean of relative standard deviation of humic acid and tryptophan between and within the devices LS 50 and LS 55. All points of an EEM are considered.

Substance and device	Median	Mean
Tryptophan 20 μg/L, LS 50	5.54	11.22
Tryptophan 20 μg/L, LS 55	6.29	12.90
Tryptophan 20 μg/L, LS 50/55	12.02	21.14
Tryptophan 100 μg/L, LS 50	2.65	6.43
Tryptophan 100 μg/L, LS 55	3.77	6.39
Tryptophan 100 μg/L, LS 50/55	10.60	14.00
Humic acid 2 mg/L, LS 50	2.44	4.1
Humic acid 2 mg/L, LS 55	3.85	3.88
Humic acid 2 mg/L, LS 50/55	8.43	10.61
Humic acid 10 mg/L, LS 50	1.50	2.32
Humic acid 10 mg/L, LS 55	2.02	2.84
Humic acid 10 mg/L, LS 50/55	6.55	7.23

matrix. If an IFE correction is desired, subtraction should be done after the IFE correction. Using buffer solutions an IFE correction of the blank is recommended because of the higher absorption in the UV range of the buffer's salt.

4. Results and discussion

4.1. Standardization procedure

The median of the relative standard deviation S_{rel} for all three repeated measurements of each solution and device, as it is shown in Table 1, are lower than 5% in most cases and will be neglected in further discussion. In Fig. 1 both the standardized, blank subtracted but not IFE corrected EEM of humic acid and tryptophan as well as the EEM of the relative standard deviation between both devices are shown.

Respectively, both matrices of one compound are very similar, considering the overall spectral shape. Nevertheless, small differences in the fluorescence intensities cannot be neglected. In order to get information about the difference for each segment of the matrix the relative standard deviation between matrices of both devices was calculated (Fig. 1(c) and (f)). Fig. 1 reveals a high level of agreement for some segments of the matrix. However, there are some regions where S_{rel} is quite high. There are differences especially in the upper peak of humic acid (at λ_{ex} = 340 nm) and tryptophan (at λ_{ex} = 220 nm). Fig. 1(c) and (f) displays that the higher values of S_{rel} are characteristic in the periphery of the matrix of humic acid and tryptophan. For both components the highest differences appear at an excitation of 220 nm. For tryptophan the standard deviation is getting higher in spectral regions of low fluorescence. It is remarkable that the level of differences between both instruments is getting to a minimum near or at peak maximum of the compounds (220/350 and 280/350 nm for tryptophan and 220/440 nm for humic acid).

The box plots of relative standard deviation versus the corrected fluorescence intensity in Fig. 2 cover all measurement points of 10 mg/L humic acid and 0.1 mg/L tryptophan. For humic acid (Fig. 2(a)) the median is lower than 10%, independent of fluorescence intensity. For tryptophan (Fig. 2(b)) the median covers a range between 10% and 20%. It is remarkable that the variance of the deviation is getting smaller, if the fluorescence intensity increases.

Fig. 3 shows the comparison of two standardized emission spectra of 0.1 mg/L tryptophan at the excitation wavelengths of 220 nm and 230 nm. It is obvious that the differences depend on the excitation wavelength. Considering the fact that the standardization procedure consists of three steps, excitation and emission correction as well as normalization, the latter cannot be the reason for these differences, because there would be a constant offset in the spectra between the two devices instead. Exemplary Figs. 4 and 5 show the effect of the single standardization steps on the emission spectra of humic acid (at λ_{ex} = 260 nm) and tryptophan (at λ_{ex} = 220 nm).

Fig. 4(a) and (b) contains the excitation corrected raw data, Fig. 4(c) and (d) the spectra after emission correction and Fig. 4(e) and (f) the Raman normalized data. It is visible, that there are big differences for the raw data in the emission between the two devices. First of all the humic acid emission spectrum shows two shoulders at λ_{em} = 450 nm and λ_{em} = 480 nm at the LS 55, whereas the LS 50 only shows one shoulder. The emission correction removes this device dependent effect. Similar results are obtained for tryptophan (Fig. 4(d)). Peak shape only fits if both corrections are performed. For a better visualization of the differences between the two devices, the spectra shown in Fig. 5 are all normalized to one. Looking at Fig. 5 it finally becomes obvious, that the emission correction works very well and produces identical emission spectra for humic acid (Fig. 5(a) and (c)) as well as for tryptophan (Fig. 5(b) and (d)).

Finally Table 1 gives an overview of the relative standard deviations. On the basis of median and mean both the differences within each device and between the two devices are shown for humic acid and tryptophan.

It is concluded that a comparability of fluorescence spectra between different instruments is given. A changing level of differences in several segments of the matrix cannot be avoided. In general, the differences are on a comparable lower level if the fluorescence is increasing, that means at the peak maximum. In areas of peaks with lower intensity and at the side of peaks the risk to obtain higher levels of difference is increasing. The reason of this effect cannot be explained in detail so far. At least the results show that the BAM kit for performing the emission correction, which eliminates deformations in the shape of emission peaks, is appropriate for application to EEM and does not lead to variations. Peak shapes are comparable after emission correction generated by the BAM kit. Applying all tools for spectra correction and normalization which are available and at identical measurement conditions, as temperature, pH-value, concentration, etc. small differences in the median between 6.6% and 12% cannot be avoided. One residual reason for these differences could be the excitation correction. In this work the manufactures excitation correction function has been used without creating an own function, e.g. by using Rhodamine 101. This obvious fact has to be considered if the fluorescence technique in this form will be introduced for online- or onsite-monitoring procedures, e.g. of waters. In case of monitoring river water or treatment trains at several points in waterworks an identical technique should be applied if these small differences determined in this work are not acceptable. Interlaboratory tests should also consider this, because differences between instruments from further companies not examined in this work are possible.

4.2. Inner filter effects

After performing the standardization procedure described in Section 2.1 and demonstrated in Section 4.1 it is recommended to also perform the correction for inner filter effects by the method of Lakowicz [12]. Taking into consideration that IFE only depend on the absorption of the sample itself the correction is independent of the used fluorescence spectrometer. This can be seen in the IFE correction equation of Lakowicz and Gauthier, which do not contain any device dependent parameters. Therefore the application of the IFE correction leads to identical variations of relative standard deviation between the two devices shown in Section 4.1. However, this correction is suitable to obtain a linear correlation between the concentration of a fluorophore and its fluorescence intensity. Therefore this procedure is an important part of the whole spectra correction if results with varying concentrations are to be compared, as it is the case for samples from drinking water treatment.

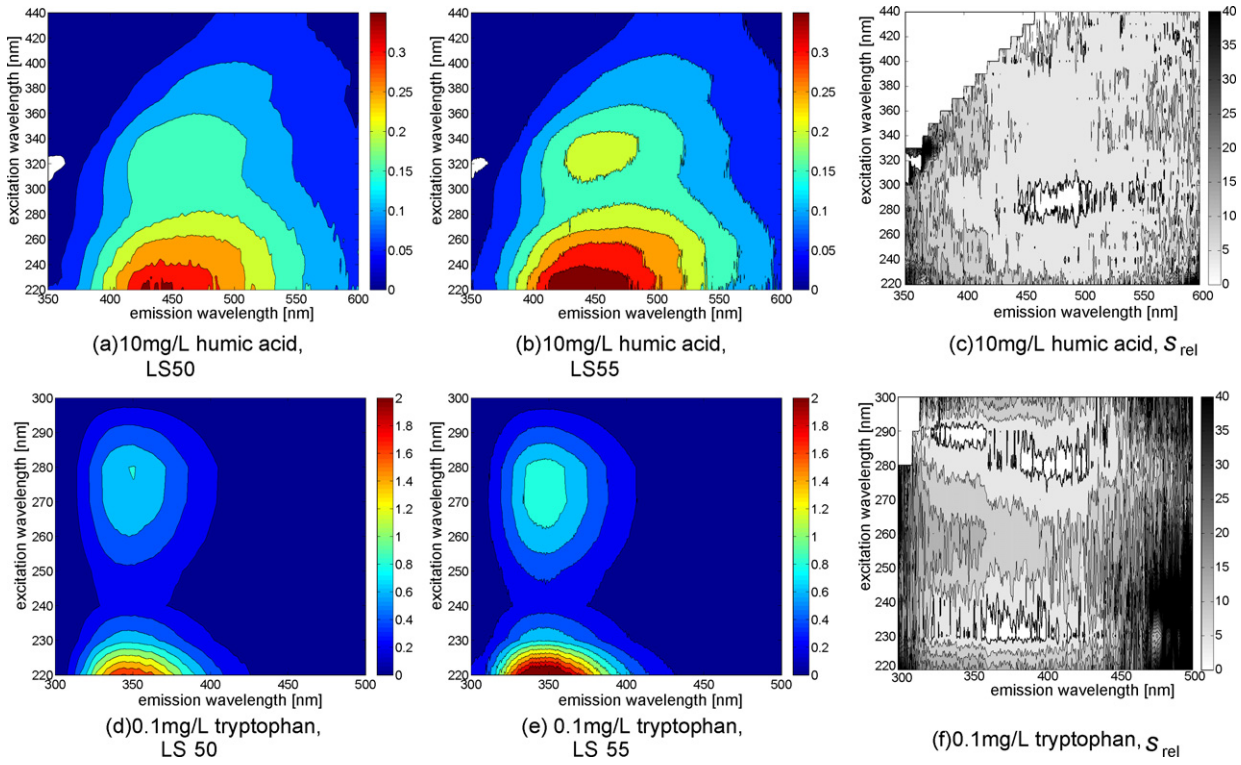


Fig. 1. EEM's of 10 mg/L humic acid and 0.1 mg/L tryptophan. Each EEM is the average of three measurements. S_{rel} shows the relative standard deviation between the two devices LS 50 and LS 55. S_{rel} in % and fluorescence intensities in r.u.

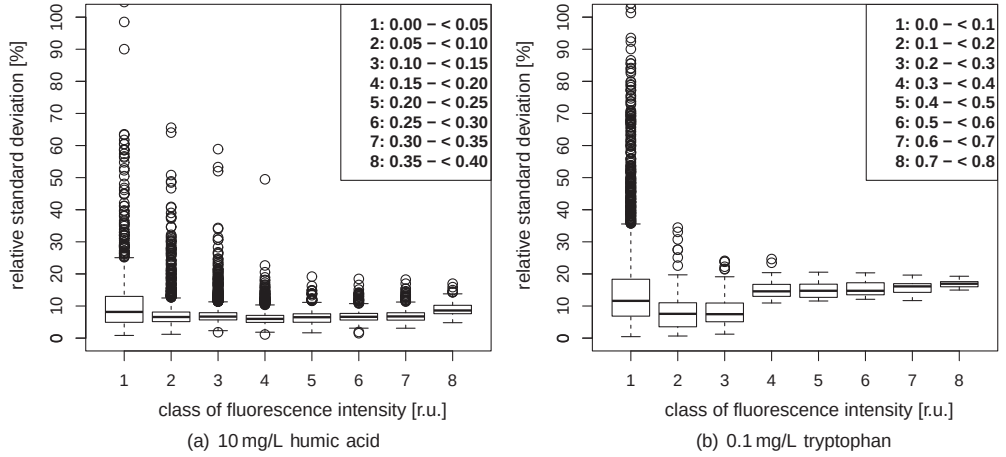


Fig. 2. Box plots of relative standard deviation versus classes of fluorescence intensities for 10 mg/L humic acid and 0.1 mg/L tryptophan.

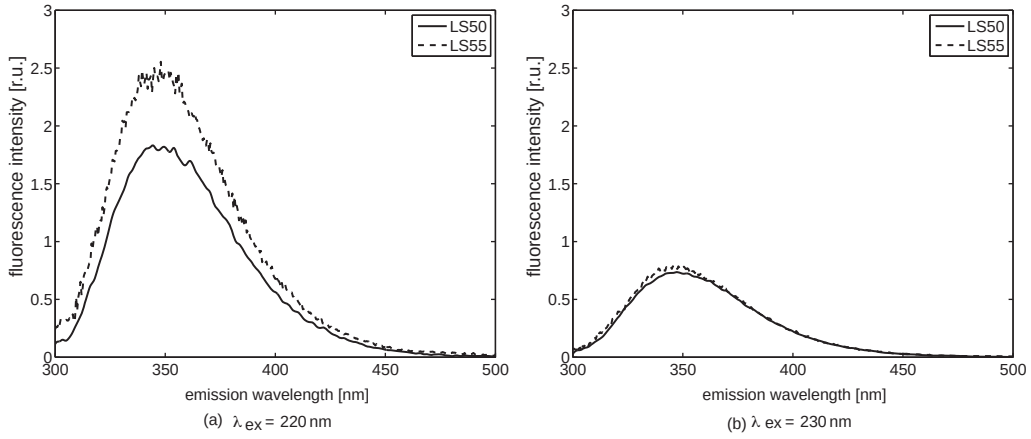
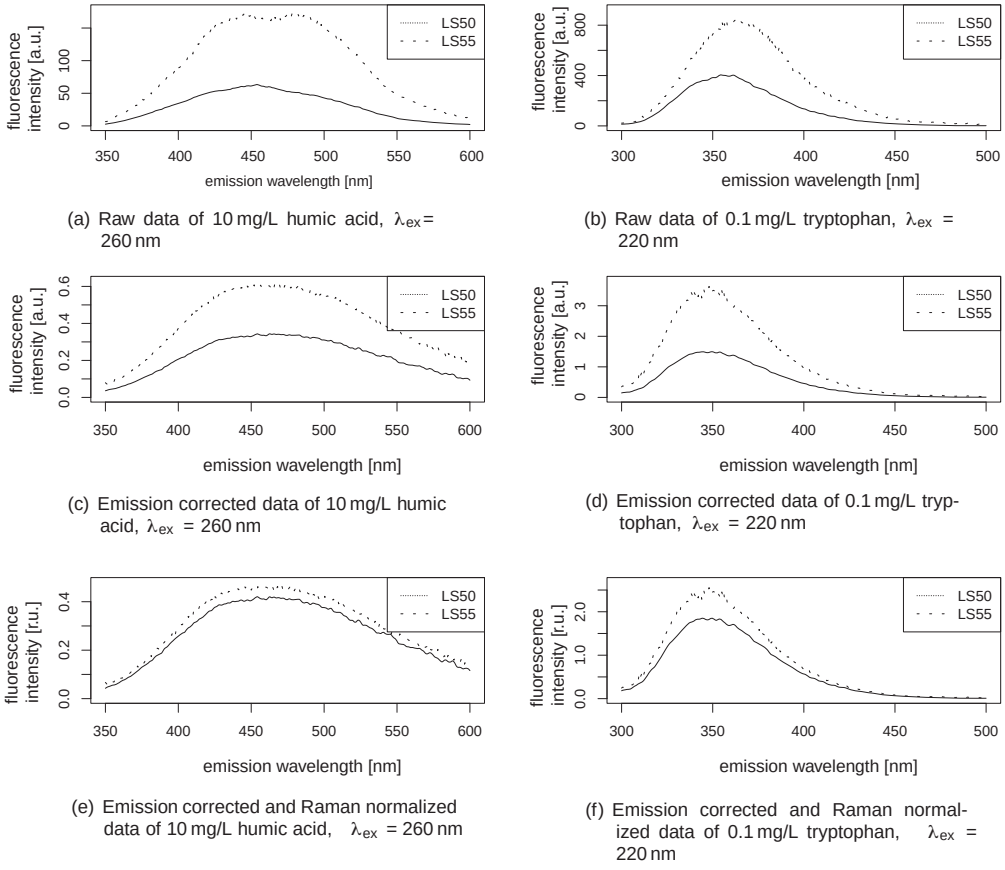


Fig. 3. Comparison of standardized emission spectra of 0.1 mg/L tryptophan at excitation of 220 nm and 230 nm.



(a) Raw data of 10 mg/L humic acid, λ_{ex} = 260 nm

(b) Raw data of 0.1 mg/L tryptophan, λ_{ex} = 220 nm

(c) Emission corrected data of 10 mg/L humic acid, λ_{ex} = 260 nm

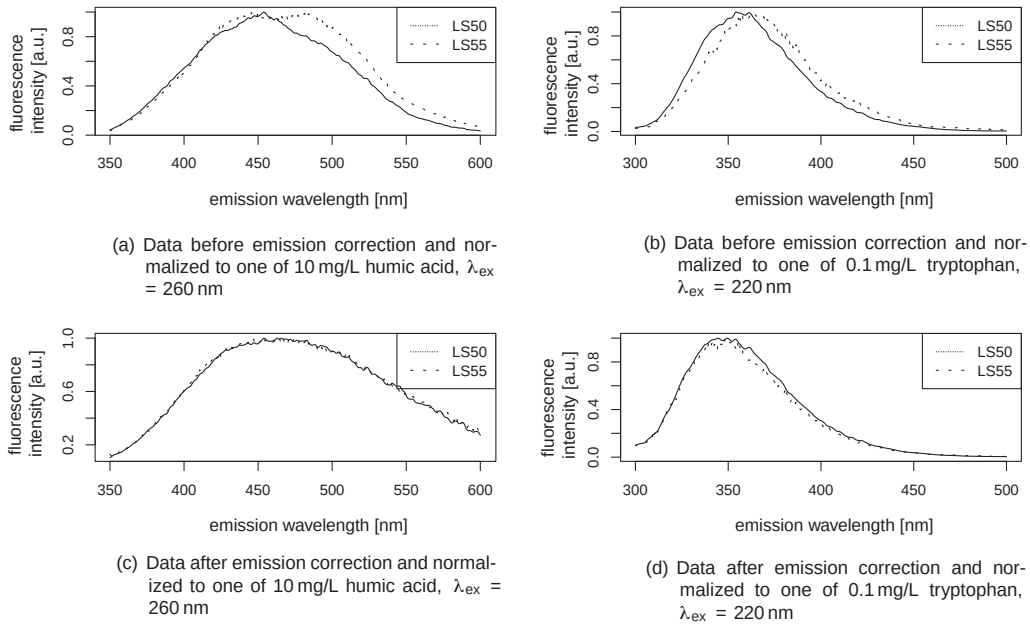
(d) Emission corrected data of 0.1 mg/L tryptophan, λ_{ex} = 220 nm

(e) Emission corrected and Raman normalized data of 10 mg/L humic acid, λ_{ex} = 260 nm

(f) Emission corrected and Raman normalized data of 0.1 mg/L tryptophan, λ_{ex} = 220 nm

Fig. 4. Step-by-step plots of the emission spectra of 10 mg/L humic acid at λ_{ex} = 260 nm and 0.1 mg/L tryptophan at λ_{ex} = 220 nm.

Table 2 shows the values for R^2 after applying inner filter effect correction by Lakowicz and Gauthier for the humic acid standard. It is not possible to apply the method by Larsson. The use of emission cut-off filters led to elimination of Raman peaks of the sample. Obviously the correction methods of Lakowicz and Gauthier result in quite similar values, which is due to their close mathematical relation. Therefore there are just small differences in the R^2 values. For the humic acid standard the correction method of Lakowicz gives the best R^2. The advantage of the Lakowicz method lies in the direct measurement of the IFEs by absorbance. Gauthier's method is similar to Lakowicz's approach but requires more parameters like cell geometry. In contrast the Larsson correction is characterized by



(a) Data before emission correction and normalized to one of 10 mg/L humic acid, λ_{ex} = 260 nm

(b) Data before emission correction and normalized to one of 0.1 mg/L tryptophan, λ_{ex} = 220 nm

(c) Data after emission correction and normalized to one of 10 mg/L humic acid, λ_{ex} = 260 nm

(d) Data after emission correction and normalized to one of 0.1 mg/L tryptophan, λ_{ex} = 220 nm

Fig. 5. Normalized raw data of 10 mg/L humic acid at λ_{ex} = 260 nm and 0.1 mg/L tryptophan at λ_{ex} = 220 nm after excitation correction and after emission correction.

Table 2
R^2 values of a linear regression at two intensities at 230/440 and 320/440 (ex/em) nm for humic acid concentrations 1–30 mg/L.

	Int 230/440	Int 320/440
Standardized without IFE correction	0.698	0.900
Lakowicz	0.995	0.987
Gauthier	0.973	0.982

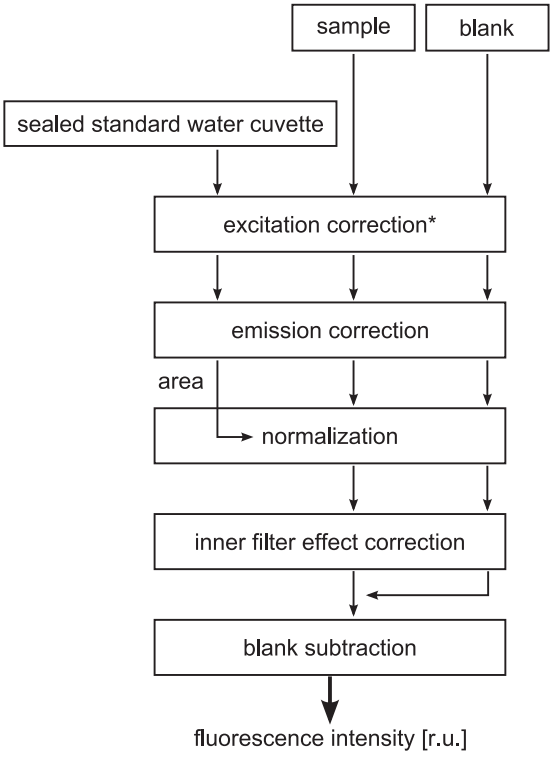


Fig. 6. Flow chart of standardization procedure; *part of the PerkinElmer spectrometer software.

the ratio between the Raman peak of the sample and of pure water. This is an indirectly way of absorbance measurement.

5. Conclusion

In this work a standardization procedure covering a spectral correction, Raman normalization and inner filter effect correction was applied to compare whole fluorescence matrices measured by two different spectrometers (Fig. 6).

The results obtained by spectral correction and normalization as well as inner filter effect correction are discussed separately.

On the one hand, if spectra are measured several times with one instrument using exactly the same conditions, the data is completely reproducible. On the other hand, if different instruments are used the spectra correction and Raman normalization cannot eliminate every differences in the spectra. In case of standard compounds and instruments used in this work the median of relative standard deviation between spectra in the protein-sector can be reduced to a nearly constant level of less than 12%. In case of humic acid the median of S_{rel} is determined to be constant lower than 10%. The higher the fluorescence the lower the variance of S_{rel} is. It is suggested that the still occurring differences are due to spectral deformation which so far cannot be corrected, however, they are assumed to be a result of excitation correction. The highest differences are obtained near the periphery of the matrix measured in this work. For example, the emission correction function is not valid for λ_{em} lower than 300 nm. Further research is needed if monitoring of fluorophores in this range, e.g. of tyrosine-like compounds, is needed.

In the segment of humic acid fluorescence, which is the most important organic matter in natural waters, the comparability of fluorescence with a standard deviation of lower 10% (median) at adequate fluorescence intensities seems to be sufficient. Nevertheless, further measurements using more different instruments and compounds should be carried out. Besides the fluorescence maxima also the whole fluorescence matrix should be considered in order to obtain a complete impression of the amount of differences which can be expected.

For the correction of inner filter effects it was shown that the method of Lakowicz should be applied as it was the best working method in this study. However, the correction procedure of Lakowicz needs absorption measurements which complicates an application in an online-procedure and may introduce further errors. Results for the method of Gauthier are similar to those of the method of Lakowicz. The procedure of Gauthier includes more constants in its mathematical description making an introduction of errors likely. All in all, if no information on any preconditions is given, the correction procedure of Lakowicz is still the best working one, as attested by other studies too [9,11]. The procedure by Larsson was not performed in this work, because of missing Raman scatter peaks by using emission cut-off filters. Nevertheless it is appropriate for low DOC concentrations and has its advantages for online devices, where absorption is not measured.

Under the aspect of drinking water control by fluorescence technique it can be concluded that there is a discrepancy between the measurement itself and the assessment of the spectra. The measurement has real time character, but the assessment of the spectra implies several mentioned difficulties which have to be considered by users. The remaining differences are to be assessed. That means in every case the decision about the comparability of fluorescence spectra requires an extensive procedure which is in contrast to the measurement itself. Therefore prospective work is necessary to establish a consistent operating procedure as presented in this work embedded in software tools for easy use.

References

[1] R.K. Henderson, A. Baker, K.R. Murphy, A. Hambly, R.M. Stuetz, S.J. Khan, Water Res. 43 (2009) 863–881.
[2] A. Baker, Environ. Sci. Technol. 35 (2001) 948–953.
[3] P.G. Coble, Mar. Chem. 51 (1996) 325–346.
[4] C.A. Stedmon, S. Markager, R. Bro, Mar. Chem. 82 (2003) 239–254.
[5] S.A. Cumberland, A. Baker, Hydrol. Process. 21 (2007) 2093–2099.
[6] S. Determann, J.M. Lobbes, R. Reuter, J. Rullkötter, Mar. Chem. 62 (1998) 137–156.
[7] D. Gondar, S.A. Thacker, E. Tipping, A. Baker, Water Res. 42 (2008) 81–90.
[8] M.Z. Bieroza, J. Bridgeman, A. Baker, Drink. Water Eng. Sci. 3 (2010) 63–70.
[9] A.J. Lawaetz, C.A. Stedmon, Appl. Spectrosc. 63 (2009) 936–940.
[10] U. Resch-Genger, D. Pfeifer, C. Monte, W. Pilz, A. Hoffmann, M. Spieles, K. Rurack, J. Hollandt, D. Taubert, B. Schönenberger, P. Nording, J. Fluoresc. 15 (2005) 315–336.
[11] K.R. Murphy, K.D. Butler, R.G.M. Spencer, C.A. Stedmon, J.R. Boehme, G.R. Aiken, Environ. Sci. Technol. 44 (2010) 9405–9412.
[12] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, 3rd edition, Springer, Baltimore, Maryland, 2006.
[13] P.G. Coble, C.E. del Castillo, B. Avril, Deep Sea Res. Part II 45 (1998) 2195–2223.
[14] K.R. Murphy, Lecture at AGU Chapman Conference on Organic Matter Fluorescence, University of Birmingham, 2008.
[15] A. Baker, Water Res. 39 (2005) 4405–4412.
[16] T.D. Gauthier, E.C. Shane, W.F. Guerin, C.L. Seitz, C.L. Grant, Environ. Sci. Technol. 20 (1986) 1162–1166.
[17] T. Larsson, M. Wedborg, D. Turner, Anal. Chim. Acta 583 (2007) 357–363.
[18] C. Eliot-Laize, P.S. Naden, G.H. Old, Lecture at AGU Chapman Conference on Organic Matter Fluorescence, University of Birmingham, 2008.