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A. J. LAWAETZ* and C. A. STEDMON

Dept. Food Science, Faculty of Life Sciences, University Copenhagen, Rolighedsvej 30, DK-1958, Frederiksberg, Denmark (A.J.L.); and Department of Marine Ecology, National Environmental Research Institute, University of Aarhus, Frederiksborgvej 399, Roskilde, 4000 Denmark (C.A.S.)

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Fluorescence data of replicate samples obtained from different fluorescence spectrometers or by the same spectrometer but with different instrument settings can have great intensity differences. In order to compare such data an intensity calibration must be applied. Here we explain a simple calibration method for fluorescence intensity using only the integrated area of a water Raman peak. By applying this method to data from three different instruments, we show that it is possible to remove instrument-dependent intensity factors, and we present results on a unified scale of Raman units. The method presented is a rapid and simple approach suitable for routine measurements with no need for hazardous chemicals.

Index Headings: **Fluorescence spectroscopy; Intensity standardization; Water Raman peak.**

INTRODUCTION

Fluorescence spectroscopy is a popular analytical method applied in a wide range of fields.[1–3] It has the advantage of being highly sensitive (down to ppb) and selective, which makes it a powerful analytical tool for both quantitative and qualitative analysis.[4] There are, however, certain drawbacks. The fluorescence signal can be instrument dependent, and therefore there is a need for standardization if fluorescence data are to be compared between instruments.

For fluorescence data there are three data correction stages that have to be considered prior to data analysis (Fig. 1). The first stage is correction for the spectral properties of the instrument (excitation and emission correction). This removes the instrument-specific spectral biases. This correction step is important and new approaches are currently being assessed and developed by the National Institute of Standards and Technology (NIST, Gaithersburg, MD) and the Federal Institute for Materials Research and Testing (BAM, Germany).[5–7] The second stage is correction for the absorption properties of the sample (often referred to as inner filter effects[4]). In optically thin samples with low absorbance, this is not necessary.[8] After completing these first two stages, data should be spectrally inter-comparable between instruments and over time. However, the intensity of the fluorescence signal is not yet calibrated, and this is done in the final stage. In the same sense as the spectral position of the peak can be influenced by the instrument, the intensity of the fluorescence signal is also very dependent on the instrument. Different instruments have different detector systems and/or use different photomultipliers; hence they often use different scales for the fluorescence intensity. Additionally, the fluorescence intensity is almost always (except for photon counting systems) given in arbitrary units (A.U.). This makes quantitative fluorescence spectroscopy difficult across different instruments. When working with well characterized and known fluorophores, this is a minor problem that can be circumnavigated using a series of concentration standards. This is, for example, done in the routine measurement of the plant pigment *chlorophyll a* in marine research.[9] However, when working with a complex mixture of potentially unknown fluorophores a different approach is required. To date the majority of fluorescence studies attempt to avoid this problem either by carrying out all measurements on the same instrument or by using an external well-characterized standard such as quinine sulfate or, more recently, using reference standard material 2941, both supplied by NIST.[10–12]

There is, however, an alternative less widespread method that uses the scattering properties of pure water as a quantitative standard. This involves the properties of the water Raman peak. The technique has been applied before[13,14] but not explained or demonstrated thoroughly or clearly in the literature. Also, applications so far are nearly exclusively found within the aquatic sciences though this method can be applied universally to a broad range of fluorescence applications. Here we explain the approach in a simple way and emphasize its utility as a straightforward and robust calibration technique. The technique is demonstrated for single excitation and emission wavelength pair fluorescence measurements, but it is equally applicable for 2d and 3d fluorescence spectra. A minimum of data are presented in order to make the presentation user friendly and to stress the simplicity of the method.

RAMAN SCATTER BAND AND THE CALIBRATION APPROACH

Pure water has two clear scatter peaks: Rayleigh and Raman (Fig. 2). The first is due to direct scattering of the incident light and therefore occurs at the same wavelength as the excitation. The water Raman peak is, however, a result of non-elastic scatter. A fraction of the incident photons lose energy to vibration in water molecules and the photon is then scattered at a higher wavelength than the incident light. The energy loss in water has a fixed frequency of approximately 3400 cm^{-1}.[15] The Raman peak has a relatively low intensity and is often overshadowed by the fluorescence of even moderate concentrations of fluorophores that fluoresce at these wavelengths.

The wavelength-dependent Raman cross-section of water is a fixed property of water and the integral of the measured Raman peak (A_{rp}) (Fig. 2) is directly proportional to it.[15] A_{rp} can therefore be used to calibrate measurements made on different instruments, or made with different instrumental settings as the peak height and width will vary accordingly. Raman peaks measured for each setup used to measure samples

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* Author to whom correspondence should be sent. E-mail: ajla@life.ku.dk.

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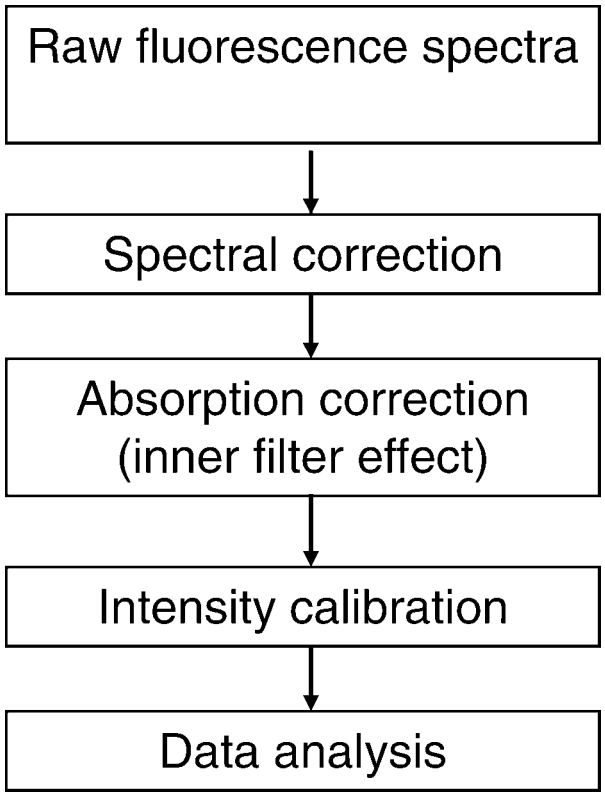

FIG. 1. Flow chart of the different correction steps for fluorescence data.

can then be used to calibrate the data accordingly. This is particularly relevant for applications in which the concentration span of the fluorophore of interest is so wide that it is necessary to change the instrument setup, or equally in situations where samples are measured at different locations. The spectral position of the Raman peak in a water-based solution for any specific excitation wavelength (λ_{ex}) can be calculated using Eq. 1 as illustrated in Fig. 2:

$$\textit{Raman peak position } [\lambda_{em}(\text{nm})] = 1 \times 10^7 \left(\frac{1 \times 10^7}{\lambda_{ex}} - 3400 \right)^{-1} \quad (1)$$

In order to calculate the integral of the Raman peak (A_{rp}) one needs to define the wavelength band over which to integrate. Assuming pure water is used, the signal on either side of the peak should be very low and within instrumental noise. As the width of the Raman peak varies depending on instrumental setup, we suggest that a relatively broad fixed band is used. This ensures that it is valid for as broad as possible a range of excitation wavelengths and instrumental parameters. We have chosen to define the Raman peak width as peak position ± 1800 cm^{-1}. For an excitation wavelength of 350 nm, this equates to a band spanning from 371 to 428 nm. Depending on applications, alternative Raman peaks (i.e., from a different excitation wavelength) may be used, but it is important to report which excitation wavelength has been used for the calibration. We suggest the Raman peak from 350 nm excitation as this is already used for signal-to-noise determinations and it is within the range of many fluorescence spectrophotometers. A_{rp} is dependent on the excitation

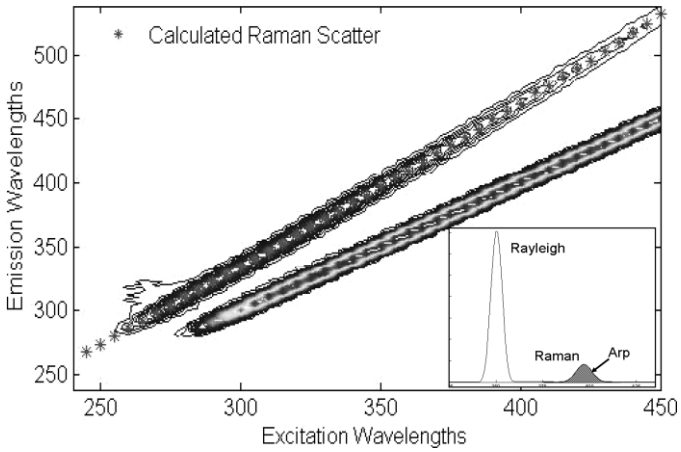

FIG. 2. Excitation–emission matrix for a MilliQ water sample showing the Rayleigh and Raman scatter bands. Also plotted are the calculated Raman peak wavelengths according to Eq. 1. Insertion is the emission spectrum at 350 nm excitation showing the Rayleigh and Raman peaks. The area under the Raman peak has been marked to illustrate the A_{rp}.

wavelength chosen and is calculated according to Eq. 2:

$$A_{rp}^{\lambda_{ex}} = \int_{\lambda_{em}^1}^{\lambda_{em}^2} I_{\lambda_{em}} \, d\lambda_{em} \quad (2)$$

I_λ is the measured spectrally corrected intensity of the Raman peak at emission wavelength λ. For practical use, A_{rp} is obtained by summing the intensity at every wavelength. It is important to note that some fluorescence spectrophotometers record emission data at intervals other than every 1 nm. This has to be taken into consideration before calculating A_{rp}.

To perform the calibration the fluorescence of a sample at any wavelength is normalized to A_{rp} determined daily for the particular instrumental setup (Eq. 3). The fluorescence signal at all measured wavelengths is now calibrated to so-called Raman Units (R.U.), which is in turn quantitatively independent of instrument specificities and therefore comparable to measurements from other instruments or from the same instrument but with different settings.

$$F_{\lambda_{ex},\lambda_{em}}(\text{R.U.}) = \frac{I_{\lambda_{ex},\lambda_{em}}(\text{A.U.})}{A_{rp}} \quad (3)$$

It is important to note that this approach differs from another commonly used Raman intensity normalization approach, in which the measured signal is normalized to the peak intensity alone rather than A_{rp} (e.g., Giana et al. (2003)[16]; Holbrook et al. (2006)[7]). Only normalizing to A_{rp} will result in a truly universal scale that should be independent of instrumental parameters, provided that spectral corrected data is used.[5,6] This will be demonstrated with some simple examples.

EXPERIMENTAL

In order to demonstrate the approach, a simple intercalibration was carried out on three different instruments. A series of concentration standards of quinine sulfate were made using quinine sulfate obtained from NIST in perchloric acid ($HClO_4$) according to the procedure from Velapoldi and Mielenz.[10] The series of concentration standards consisted of concentrations of 0, 1, 2.5, 5, and 7.5 ppb. Fluorescence

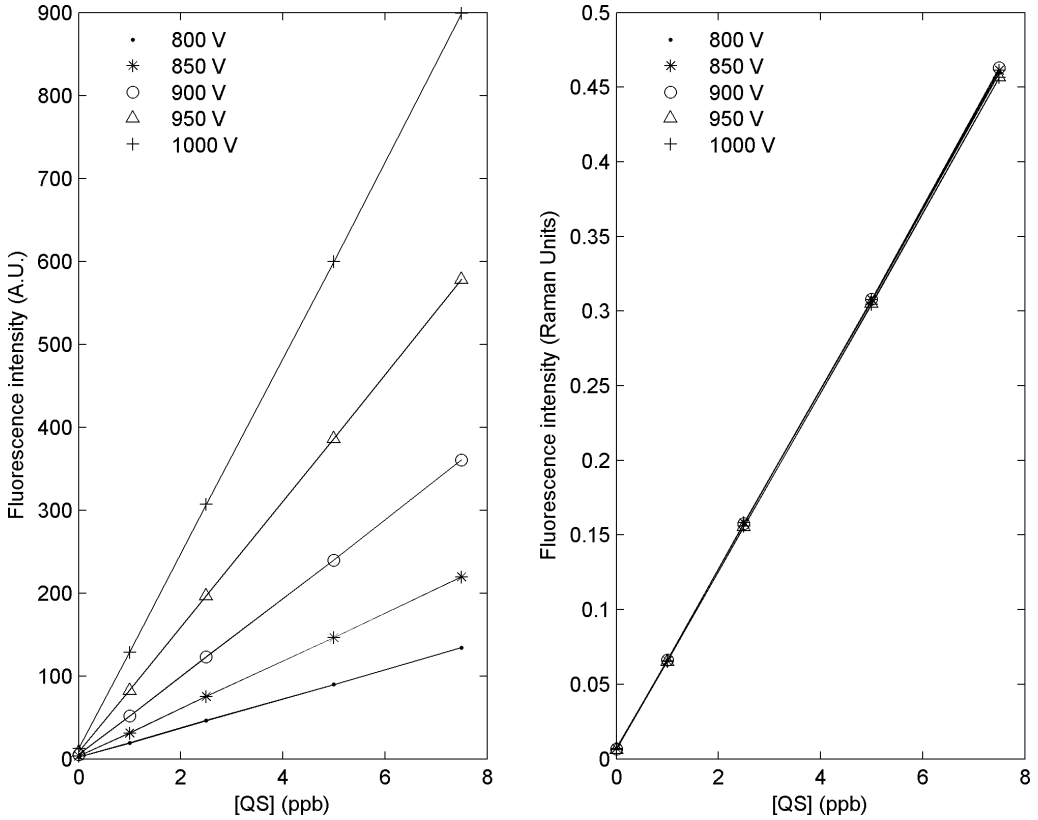

FIG. 3. Fluorescence of quinine sulfate at different concentrations measured using variable photomultiplier tube (PMT) voltages on a Varian Cary Eclipse. (**Left**) before and (**right**) after Raman calibration. Raman units can easily be converted to QS-equivalents by the equation QS = RU/0.0767.

measurements were carried out on a Varian Cary Eclipse, an LS 55 Perkin Elmer, and an FS920 Edinburgh Instruments fluorescence spectrophotometer. For all measurements excitation and emission slits were set to 5 nm. Water Raman spectra were recorded with an excitation wavelength of 350 nm and emission wavelengths from 365 to 430 nm. Fluorescence of quinine sulfate (QS) was measured at an excitation wavelength of 250 and emission wavelength of 450 nm. The photomultiplier tube (PMT) voltage was varied during the measurements. For the Varian, the voltages used were 800, 850, 900, 950, and 1000 V, and for the LS55 700, 750, 800, 850, and 900 V were used. The FS920 is a photon-counting instrument and hence the detector voltage could not be varied. All the samples (QS solutions and water samples) were measured using the different instrument setups. All samples were measured in replicates of at least five and the mean value was calculated and used for the data analysis. The whole experiment was repeated a month later with a new batch of QS standards. Additionally, spectra of a single sample were measured (ex. 250 nm, em. 300–600 nm) on the Varian with different excitation and emission slit width settings (5/5, 10/5, 2.5/5, 1.5/5, and 5/2.5 nm). Five parts per billion (5 ppb) QS and detector voltage of 950 V was used for these measurements. Emission spectra were corrected for the wavelength-dependent spectral bias using a correction factor derived by use of secondary emission standards provided from BAM.[5] In addition, during measurement the source intensity in all three instruments was normalized to that of an internal reference detector.

RESULTS AND DISCUSSION

As stated above, sometimes it can be necessary to change the instrument setup in order to obtain the best spectra from a set of samples. The effects of this and the result of the subsequent Raman calibration are illustrated in Fig. 3. A greater detector voltage results in a greater fluorescence signal on the same solution (Fig. 3, left panel). This makes quantitative comparison of the measurements impossible. Applying Raman calibration to the data removes these differences and places all measurements on an equal scale of Raman units (Fig. 3, right panel). It is now straightforward to make quantitative judgments of the fluorophore of interest across measurements with different instrument setups.

The instruments applied in this study were from three different manufactures, and they use different techniques for detecting the fluorescence signal. The FS 920 from Edinburgh Instruments is a photon-counting instrument, whereas the two other instruments use an arbitrary scale from 0 to 1000, but these are not equally calibrated. This will, of course, give three different results in terms of intensity, thereby making inter-instrumental comparison impossible without applying intensity calibration. The Raman calibration applied here removes these instrument-specific intensity factors and is thus suitable for such inter-instrument intensity calibration. The results of two series of concentration standards of QS measured on two different days on the three instruments after Raman calibration are shown in Fig. 4. Put on a Raman unit scale, the same concentration of QS gives the same intensity independent of instrument.

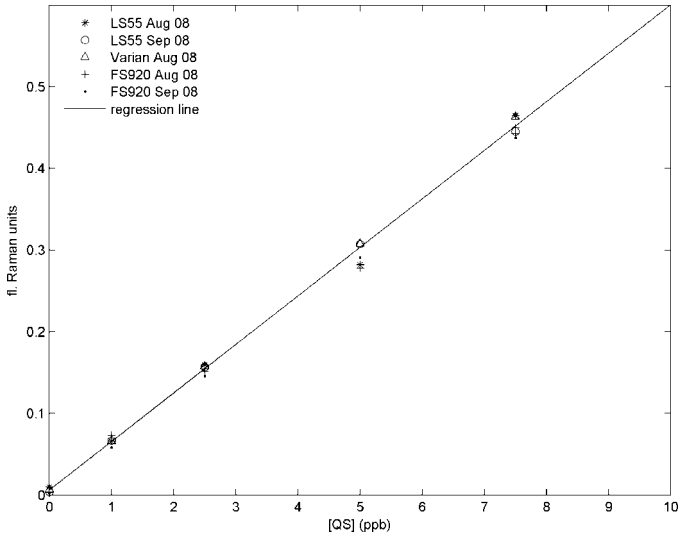

FIG. 4. Raman corrected fluorescence intensity of a standard series of quinine sulfate. Two identical series are measured on two different days, on three different instruments.

In the above example we have varied the PMT voltage of the instruments, but as illustrated in Fig. 5 similar results are obtained if excitation and emission slit widths are varied instead. Different slit widths result in different intensities of the spectrum, but after Raman correction the intensities are equal. Of course, changing the slit widths can in extreme cases change the spectral shape of the peak. Especially if there are narrow and well defined peaks, some of the resolution will be lost. This is of course not handled by the Raman correction. The Raman corrected spectrum with slit settings 1.5/5 is noisy due to the fact that both the Raman peak and the raw spectrum have low intensity and, hence, have a low signal-to-noise ratio. This is a general limitation of the method: if the instrument setup applied does not allow measuring a Raman peak with a proper signal-to-noise ratio, too much noise will be introduced to the normalized spectra and the method will not work as well as intended. In some situations, using a Raman peak of a lower excitation wavelength can solve this problem, as it will give a better signal-to-noise ratio on the Raman peak. However, in general the 350 nm excitation Raman peak is suitable. There is a fixed relationship dependence between A_{rp} from different excitation wavelengths,[15] which makes it possible to recalibrate to different Raman peaks, should it be required.

The above results reveal that Raman calibration is a suitable tool for calibrating fluorescence measurements onto a "global" scale that makes it possible to quantitatively compare measurements from different settings on one instrument or between instruments. This calibration method is not only applicable for single excitation and emission wavelength pair fluorescence measurements (Fig. 3) but is also valid for spectral measurements (Fig. 5) as well as excitation–emission matrices (EEMs). Equation 3 is valid for all excitation–emission wavelength pairs measured, hence the subscripts λ_{ex} and λ_{em}. The integral of one, fixed, Raman peak (A_{rp}) is used to normalize the whole spectrum or EEM.

A major advantage of this approach compared to other calibration methods, such as the quinine sulfate method, is that no standards are required, thus removing operational steps (weighing, dilution, etc.), and the risk of degradation of the chemicals, which all can cause errors. The Raman approach involves no hazardous chemicals and requires only pure water (preferably deionized and ultraviolet exposed), which is available in most laboratories. To simplify the approach further, sealed cuvettes (cells) with pure water are also available from most instrument manufacturers and are suitable for this approach. In Fig. 6 a plot of three water Raman peaks is shown; one is from a fresh MilliQ sample, whereas the other

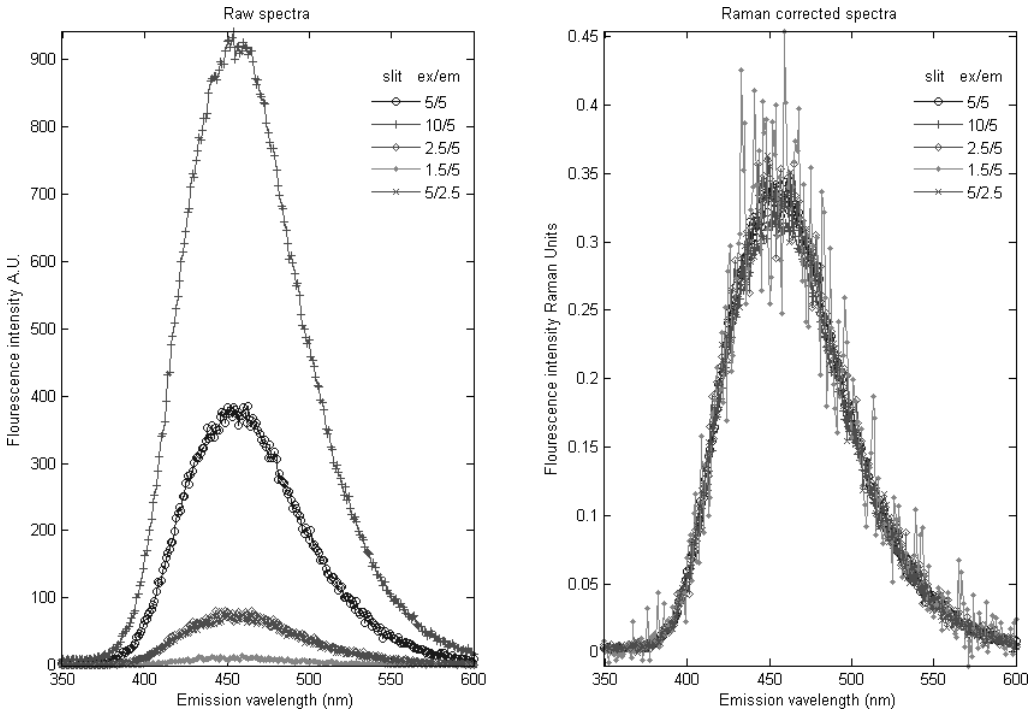

FIG. 5. Spectra of quinine sulfate solution obtained at different slit settings (**left**) before and (**right**) after Raman correction.

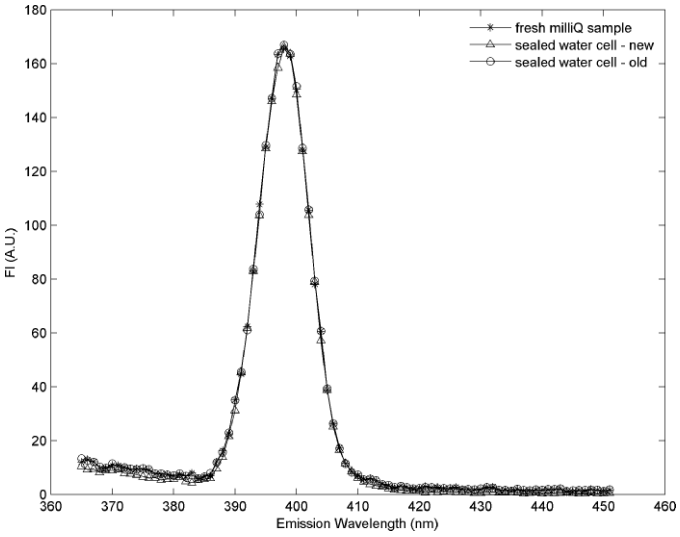

FIG. 6. Water Raman peak from a fresh MilliQ sample and two sealed water cells, a new and an old; the old one is approximately 10 years old.

two are obtained from sealed water cells. The three spectra are more or less identical (coefficient of variation for $A_{rp} < 2\%$), even though one of the sealed water cells is 10 years old. The sealed water cell can thus ensure a uniform water quality every day for a long period of time. It should also be noted that this calibration procedure is universal, and the Raman signal of water can be used irrespective of sample solvent/matrix.

Fluorescence intensity differences, inter-instrumental or due to instrument setup, can be calibrated for using the integral of the water Raman peak (A_{rp}). By Normalizing all fluorescence data to the integral of the Raman peak from excitation at 350 nm we have shown that it is possible to calibrate fluorescence data onto a global scale of Raman Units. Hereby we enable a direct comparison of fluorescence intensity from different instruments or from the same instrument using different instrumental settings and over time. It is important to stress that this is only an intensity normalization/calibration procedure. No spectral changes occur from applying this method. The problem of spectral correction should, if necessary, be addressed as an independent operation prior to the intensity calibration.[5–8]

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