Application Note

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Use of Variable Bandwidth Monochromators for Quantification of Fluorescent Probes in Produced Effluent Water

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Introduction

One of the primary waste products during the separation of oil, gas, and water is produced effluent water, which is typically a mixture of formation and injection process water containing oil, salts, chemicals, solids, and trace metals. Monitoring oil content in water streams from oil production and refining is critical to meet stringent regulations for returning produced water to the environment, and is regulated by the Environmental Protection Agency (EPA) in the US. As the current trend is to further reduce the level of crude oil released in produced water streams, more advanced methods are necessary to accurately detect minute traces of oil in purified samples, many times reaching the parts per million (ppm) or parts per billion (ppb) level.

A number of reference methods have historically been used by the industry to measure oil in produced water. These methods include infrared absorption, gravimetric and gas chromatography, and flame ionization detection (GC-FID) methods. However, none have been universally adopted, and all are subject to limitations. While infrared methods are easily used, and can be deployed in the field, they involve the use of solvents that have been shown to be carcinogenic or harmful to the environment. Gravimetric methods are inexpensive and also easy to use, but do not provide details as to the composition of the sample and are subject to evaporative constraints. GC-FID has none of the problems of the previous methods. Yet, it also has not been widely accepted due to the need for sophisticated instrumentation and a high degree of training for potential operators.

Newer methods, which incorporate a fluorescent probe, can alleviate the limitations of each of the previously listed methods, and also deliver a high level of sensitivity. This type of probe can also be used to monitor other pollutants, provided that the dye molecule has a high partitioning coefficient into the contaminant. The probe, introduced into the crude oil, is diluted during the purification process. Upon completion, a sample can easily be monitored using microplate reader technology without the need for harmful solvents or complicated procedures. As new probes are evaluated for potential introduction into the monitoring process, it is important to first understand the excitation and emission spectra of the fluorescent molecule, and then be able to tune the optical system to deliver the highest degree of sensitivity suitable for detection of the fluorescent probe when heavily diluted.

This application note demonstrates the ability to record excitation and emission spectra for fluorescent probes used in produced effluent water using the monochromator-based detection system of a microplate reader. The spectra provide the ability to begin the optimization process of wavelength and bandwidth selection for both excitation and emission monochromators. Optimization is demonstrated for two individual probes with either a narrow (23 nm) or wide (67 nm) Stokes shift. The flexibility of the monochromator-based system also allows multiple fluorescent probes, and therefore multiple potential pollutants in the final water stream to be assessed.

Materials and methods

Materials

Fluorescent contaminant probes

Unknown purified fluorescent contaminant probes 201 and 204 were donated by Sintef (Trondheim, Norway). Stock concentrations of each probe were 1 mg/mL, or 1,000 ppm, in 100% methanol.

Agilent BioTek Synergy Neo2 multimode reader

The Agilent BioTek Synergy Neo2 multimode reader is designed for speed and ultra-high performance, incorporating patented Agilent BioTek Hybrid Technology. Independent optical paths accommodate diverse assay requirements with variable bandwidth quadruple monochromators, sensitive filter-based optics, laser-based excitation for Alpha assays, and up to four PMTs for ultra-fast measurements. Advanced environment controls, including CO_2/O_2 control, incubation to 65 °C, and variable shaking are ideal for live cell assays; cell-based detection is optimized with direct bottom illumination. Barcode-labeled filter cubes help streamline workflows and limit errors.

Methods

Creation of fluorescent probe titration

A solution made up of 70% methanol/30% 0.2 M sodium hydroxide was created for the dilution of each probe. Stock solutions were initially diluted from the original 1,000 ppm concentration to 1,000 ppb. A serial titration was then performed using a 1:2 dilution scheme, creating an 11-point titration series ranging from 1,000 to 1 ppb, in addition to a diluent-only blank control.

Spectral scanning and fluorescent monochromator-based microplates reads

A single well containing the 1,000 ppb sample was used to perform an excitation and emission scan of the unknown fluorescent probe to determine the spectral characteristics. The entire titration range was then read multiple times using the variable bandwidth monochromators, while varying the excitation and emission settings, and bandwidth. The process was performed for each sample provided.

Signal-to-noise ratio and limit of detection determination

The signal-to-noise ratio (S/N) was calculated using the following formula:

$(RFU_{Probe} – Avg\ RFU_{Blank})/Std\ Dev_{Blank}$

RFU_{Probe} is the fluorescent signal from wells containing the probe titration, Avg RFU_{Blank} equals the average signal from wells containing diluent only, and Std Dev_{Blank} equals the standard deviation of the RFU values from the same blank wells. The limit of detection (LOD) is calculated as the lowest probe concentration yielding an S/N ratio of 3, or a blanked RFU value three times the standard deviation of the blank.

Results and discussion

Fluorescent probe spectral profile

The first step in the process was to accurately determine the excitation and emission spectra of the unknown sample 201. Spectral scan read steps were created in the Agilent BioTek Gen5 microplate reader and imager software, and carried out using 1 nm increments to completely capture the shape and intensity of both spectra.

Raw RFU values were captured at each wavelength tested. The values were then normalized by finding the wavelength yielding the highest RFU value, and dividing the other RFU values by the maximum. Percent maximum RFU (% max RFU) values were then plotted for each included wavelength value to create the two plots shown in Figure 1. A Stokes shift of 23 nm was determined for the probe.

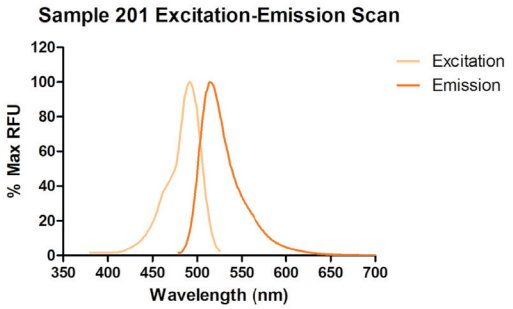

Figure 1. Sample 201 excitation and emission spectra.

Sample 201 monochromator setting optimization: on-peak assessment

To detect the lowest levels of crude oil or other pollutants in a purified water sample, limits of detection for fluorescent probes are required to be at the single ppb level or below. Therefore, monochromator optimization was carried out to determine the most ideal excitation and emission setting, in addition to bandwidth value, to meet the established criteria.

The first step was to set the excitation and emission values in a read step at the previously determined spectral peaks for the probe (on-peak). Multiple bandwidth values were evaluated from the minimum wavelength range of 3 nm to the maximum of 50 nm (Figure 2).

Wells containing the probe concentrations, diluted as previously described, were then read in a 96-well black plate using 10 measurements per data point and a delay after plate movement of 100 milliseconds.

The mean of diluent-only samples was subtracted from probe-containing well RFUs to create a blanked value. This number was then divided by the calculated standard deviation value from diluent wells to provide a S/N ratio for each concentration of probe tested. Average S/N values were then plotted using a log:log scale in relation to the known [ppb] (Figure 3).

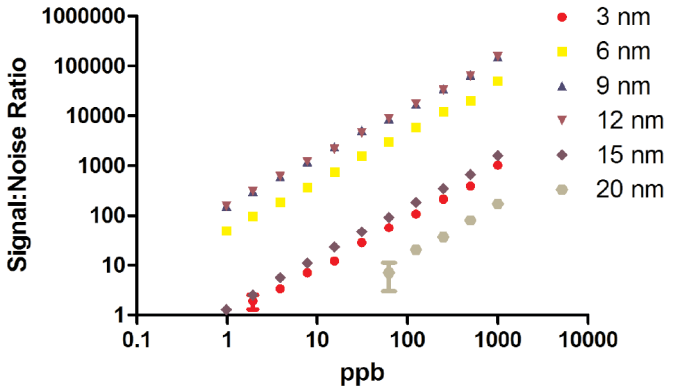

Figure 3. On-peak sample 201 calculated signal-to-noise ratio values. Excitation and emission set to determined peak values of 494 and 517 nm, respectively. S/N values incalculable for 30, 40, and 50 nm bandwidth settings.

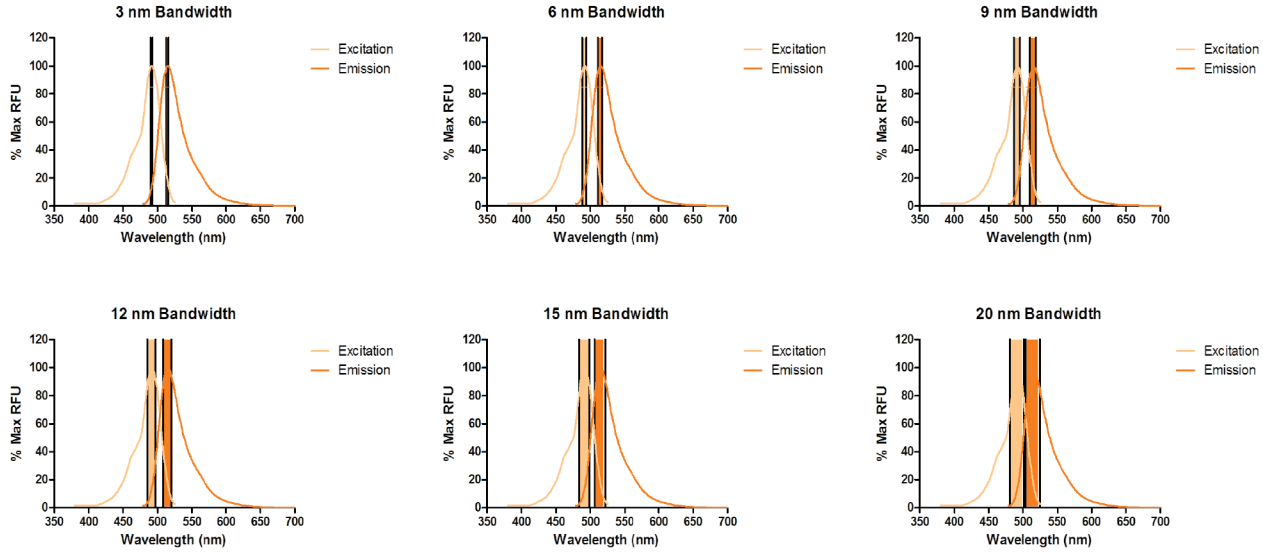

Figure 2. Sample 201 on-peak variable bandwidth wavelength coverage. Included wavelengths are shown when excitation and emission bandwidths are set to 3, 6, 9, 12, 15, or 20 nm. Bandwidths are not shown for graphs for 30, 40, and 50 nm because excessive wavelength overlap lead to crosstalk, with excitation light being captured by the emission monochromator.

As shown in Figure 3, S/N ratios for all concentrations tested increase as bandwidth expands to include a larger wavelength range, up to a value of 12 nm. This is due to the increasing amount of light used to excite the fluorescent molecule, as well as the increased range of fluorescence captured during emission. There is also minimal crosstalk, leading to minimal background levels. However, when using bandwidth values larger than 12 nm, an increasing amount of crosstalk is captured, causing background fluorescence to increase and lowering S/N ratios. Eventually, with the use of bandwidth values of 30 nm and above, the background signal is so great that fluorescence signals can no longer be quantified accurately.

The results from Figure 3 are confirmed when examining the LOD values for the bandwidths tested (Table 1). The lowest LOD is seen when a bandwidth of 9 or 12 nm is selected. It is also apparent that increased background fluorescence begins to significantly affect the sensitivity of the read beginning at a bandwidth of 20 nm.

Table 1. Sample 201 on-peak limit of detection values. Excitation and emission values set at 494 nm and 517 nm, respectively.

Bandwidth (nm)	LOD (ppb)
3	3.09
6	0.06
9	0.02
12	0.02
15	1.93
20	17.8

If a desire exists to set the excitation and emission value for the read at the determined spectral peaks, a bandwidth of 12 nm can be incorporated to yield the highest S/N ratio, the lowest LOD, and more than sufficient sensitivity for produced water effluent analysis.

Sample 201 monochromator setting optimization: off-peak assessment

While setting excitation and emission values at the spectral peak may be desired by the end user, it may not always yield the highest S/N ratios and most sensitive read parameters. This is particularly true when the fluorescent molecule has a narrow Stokes shift, leading to a larger overlap between excitation and emission spectra, as seen with sample 201. It is important to test settings that minimize unwanted crosstalk, but also allow the most optimal inclusion of wavelength ranges that will create the most sensitive read possible for the fluorescent probe.

In the case of sample 201, parameters were tested such that the highest included excitation wavelength and lowest included emission wavelength were fixed at the peak determined values of 494 and 517 nm, respectively, in accordance with the 23 nm Stokes shift. The set excitation and emission values were then moved appropriately to the left and right off of the peaks to allow incorporation of wider bandwidth ranges without crosstalk (Figure 4).

S/N ratios were once again calculated as previously described. Upon examination of the results in Figure 5, it is obvious that the same pattern is seen as when performing the on-peak optimization. Allowing an increased amount of light to excite the sample, along with capturing a larger range of the emission signal increases S/N ratios. A maximum is reached using a 20 nm bandwidth, wherein use of larger bandwidths begins to suffer increased background, lowering S/N ratios. However, what is also clear is that by minimizing the capture of unwanted crosstalk, background levels are further diminished, compared to the use of excitation and emission settings on the spectral peaks. S/N ratios can reach as much as ten-fold higher than what could be achieved during the previous test.

Higher S/N ratios yield LOD values at the ppt level that are up to 10x lower than those attained using on-peak monochromator settings (Table 2). The results of the complete set of reads performed using sample 201 confirm that LOD requirements can be met and easily exceeded by approximately one thousand fold using appropriately optimized monochromator settings, even when incorporating a fluorescent probe with a narrow Stokes shift.

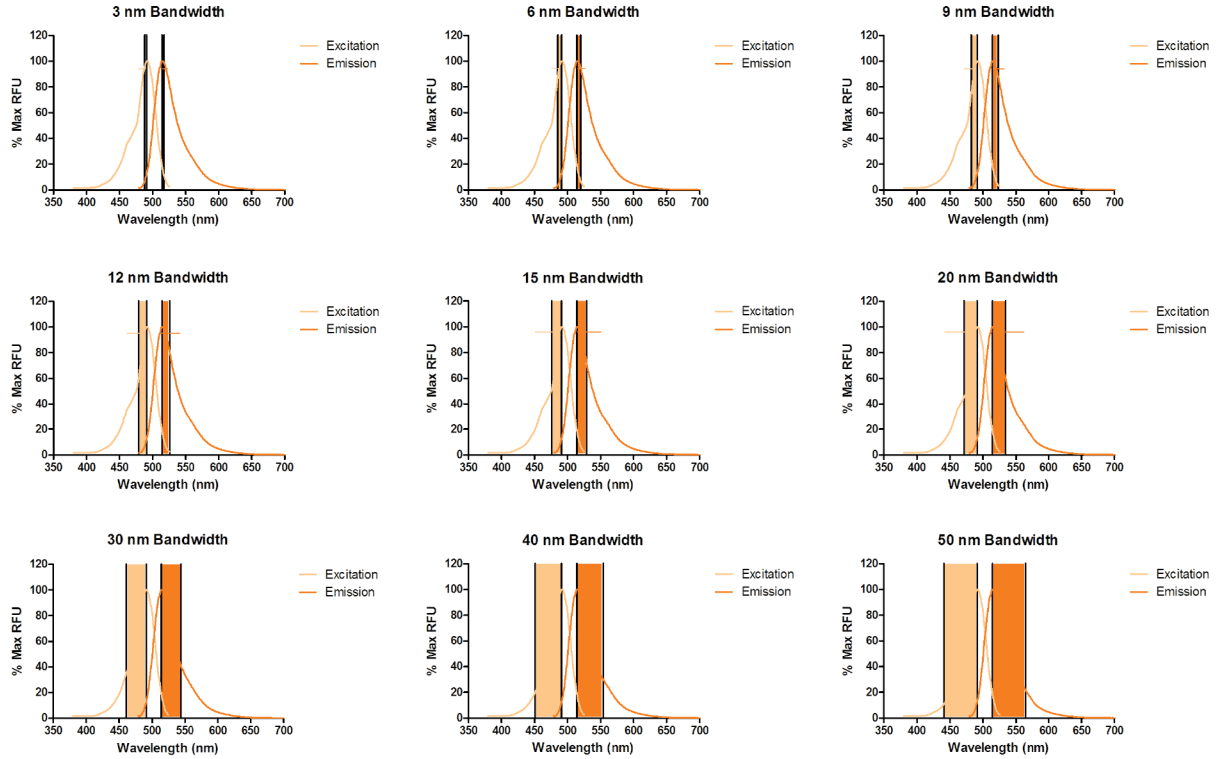

Figure 4. Sample 201 off-peak variable bandwidth wavelength coverage. A constant 23 nm gap was maintained between included excitation and emission wavelengths. Wavelength coverage is shown when excitation and emission bandwidths are set to 3, 6, 9, 12, 15, 20, 30, 40, and 50 nm bandwidths.

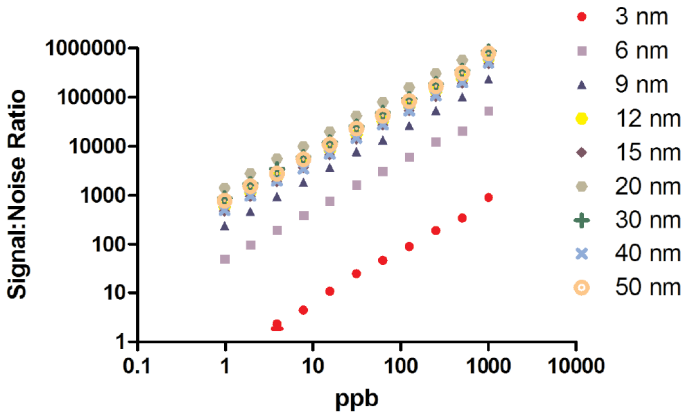

Figure 5. Off-peak sample 201 calculated signal-to-noise ratio values. Excitation and emission values were set appropriately to maintain a 23 nm gap between included wavelengths and incorporate bandwidths ranging from 3 to 50 nm.

Table 2. Sample 201 off-peak extrapolated limit of detection values.

Bandwidth (nm)	Excitation (nm)	Emission (nm)	LOD (ppt)
3	492	519	3,490
6	491	520	60
9	489	522	10
12	488	523	5
15	487	525	7
20	484	527	2
30	479	532	4
40	474	537	6
50	469	542	4

Sample 204 fluorescent probe spectral profile

A second sample was also assessed to determine the optimum monochromator settings. A spectral scan was again carried out using 1 nm increments to capture the shape and intensity of both spectra (Figure 6).

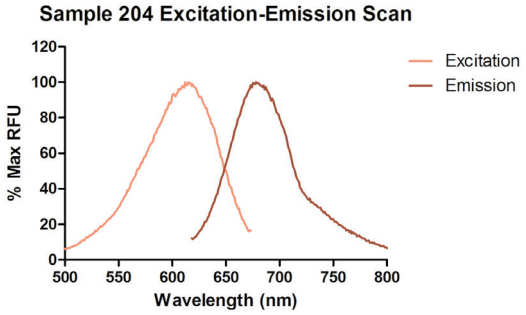

Figure 6. Sample 204 excitation and emission spectra.

When plotting the normalized signal from the spectral scans, it can be seen that as opposed to sample 201 whose spectral curves contained steep shoulders and a narrow Stokes shift, the curves for sample 204 contain much broader shoulders and a wide Stokes shift of 67 nm.

Sample 204 monochromator setting optimization: on-peak assessment

Excitation and emission values in a read step were set at the previously determined spectral peaks for the probe, 616 nm and 683 nm, respectively. Multiple bandwidth values were once again evaluated from the minimum wavelength range of 3 nm to the maximum of 50 nm (Figure 7).

Sample 204 was diluted in an equivalent manner as the previous sample, and read in the same 96-well black plate type using 10 measurements per data point and a delay after plate movement of 100 milliseconds.

Upon calculation and plotting of the average S/N values in relation to the known [ppb] (Figure 8), it is evident that expanding bandwidth increases S/N ratios until a plateau is reached with the incorporation of a 30, 40, or 50 nm range. This can be attributed to the wide Stokes shift of the fluorescent probe. This allows areas of the excitation and emission curves yielding the highest percent fluorescence to be included in the read step without the concern of increasing background signal due to capture of high levels of unwanted crosstalk, as seen with sample 201.

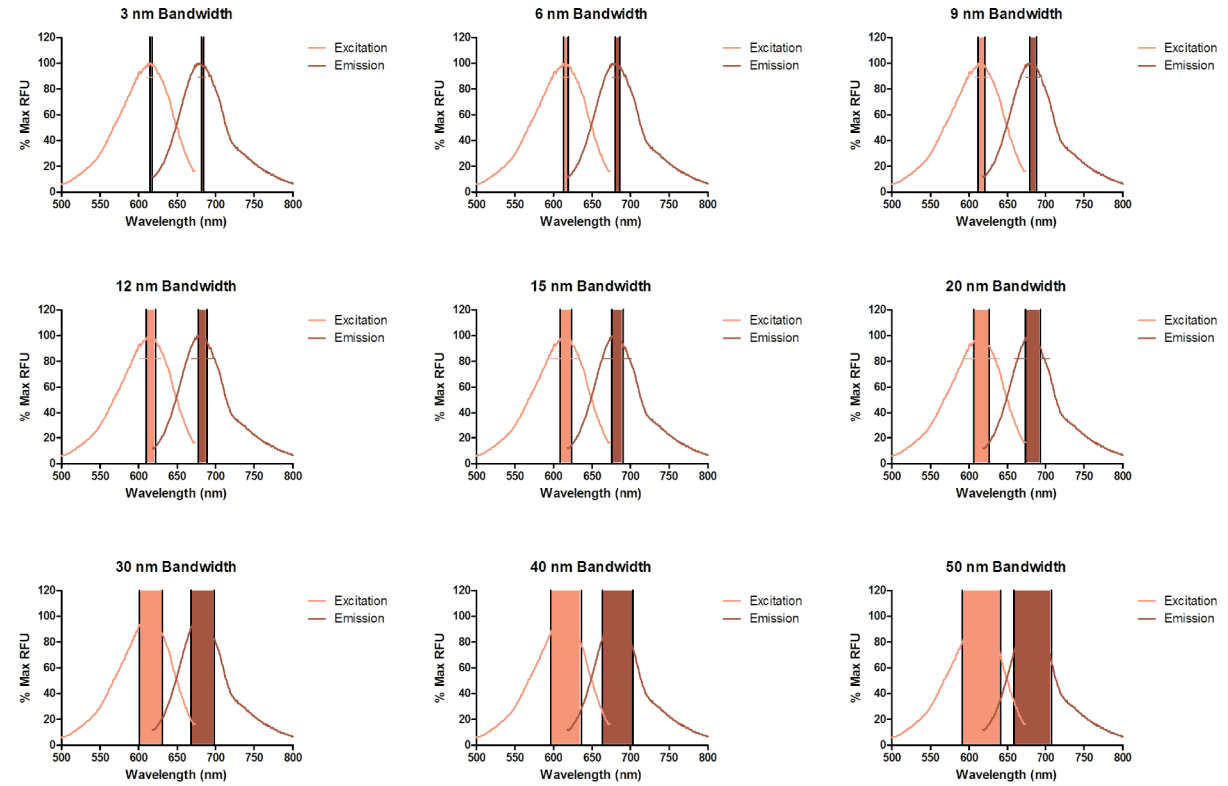

Figure 7. Sample 204 on-peak variable bandwidth wavelength coverage. Included wavelengths are shown when 616 and 683 nm excitation and emission values are chosen, respectively, and bandwidths are set to 3, 6, 9, 12, 15, 20, 30, 40, or 50 nm.

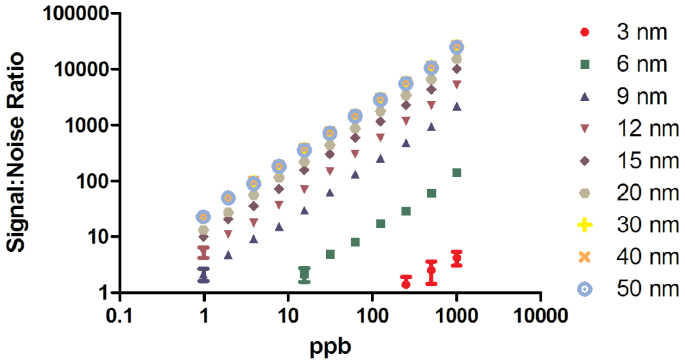

Figure 8. On-peak sample 204 calculated signal-to-noise ratio values. Excitation and emission were set to determined peak values of 616 and 683 nm, respectively.

The extrapolated LOD values in Table 3 confirm that the reads demonstrating the greatest sensitivity incorporate 30 to 50 nm bandwidths.

Table 3. Sample 204 on-peak limit of detection values. Excitation and emission values set at 616 nm and 683 nm, respectively.

Bandwidth (nm)	LOD (ppb)
3	661
6	21.8
9	1.14
12	0.59
15	0.30
20	0.20
30	0.12
40	0.12
50	0.13

Due to the wide Stokes shift and low level of crosstalk, it is not necessary to move excitation and emission settings off of the determined peaks for the fluorescent probe. The results demonstrate that monochromator settings can be optimized, this time using a fluorescent probe with a wide Stokes shift, that exceed established LOD requirements.

Conclusion

The adoption of fluorescent probes to detect trace crude oil levels and other potential pollutants in effluent water streams can greatly simplify analysis procedures, and deliver levels of sensitivity not attainable with previous methods. With the addition of a monochromator-based microplate reader, which incorporates variable bandwidth monochromators, the process can be enhanced to an even greater degree. Monochromator-based detection allows levels of multiple pollutants to be determined simultaneously, without requiring changes to the reader setup. The variable bandwidth feature then enables numerous levels of optimization to be performed to find the ideal settings for each fluorescent probe to ensure that the most robust, sensitive read parameters are achieved.

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