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DEVELOPMENT OF A MODULAR, INDUCED FLUORESCENCE-BASED INSTRUMENT FAMILY – THE AQUAFLUOSENSE PROJECT

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Abstract

In the scope of our recently completed AQUAFLUOSENSE research and development project, the design and construction of a prototype of an electron excitation fluorescence-based analytical instrument family has been carried out for water quality measurement applications. The objective of the project was to develop a new water analysis system for natural and artificial waters, allowing complex, systematic and for main parameters in situ assessment and monitoring of water quality, by developing a modular instrument family that can be individually configured for target tasks at each monitoring point. Within the instrument family, created in a collaboration of working groups of a number of research and development institutions, different modules allow for the determination of key water parameters. A common optical characteristic of these modules is that they measure the target parameter on the basis of an induced (excitation) fluorescence signal generated in the test sample. The modules allow the determination of individual biological or chemical components based either on measuring their fluorescence directly (direct fluorescence) or by relying on detection of the fluorescence of a coupled dye (indirect fluorescence). Thus, the instrument modules provide experimental data on the algal density and the total organic carbon content, as well as the presence of certain organic micropollutants in the given water body studied, the latter target analytes detected by direct fluorescence measurement or by an immunofluorescence measurement modality.
Keywords: *fluorescence, water analysis, microcontaminants, zearalenone, glyphosate*

AZ AQUAFLUOSENSE PROJEKT – MODULÁRIS, INDUKÁLT FLUORESZCENCIÁN ALAPULÓ MŰSZERCSALÁD KIFEJLESZTÉSE

Összefoglalás

A közelmúltban zárult AQUAFLUOSENSE kutatási-fejlesztési projekt keretében, elektrongerjesztett fluoreszcencián alapuló analitikai mérőműszercsalád prototípusának tervezését és kidolgozását végeztük el vízminőségmérési alkalmazásokra. A projekt célja új vízanalitikai rendszer kidolgozása volt természetes és mesterséges vizek vízminőségének komplex, szisztematikus és fő paramétereiben in situ minősítését és monitorozását lehetővé tevő, modulrendszerű, az egyes monitorozási pontokon jelentkező célfeladatokra egyedileg konfigurálható mérőműszer-család kifejlesztésével. A számos kutató és feljesztő intézmény munkacsoportjainak együttműködésében létrehozott műszercsaládon belül különféle modulok teszik lehetővé egyes vízparaméterek meghatározását, mely modulok közös optikai jellegzetessége, hogy a célparamétert a vizsgálati mintában létrehozott, gerjesztéses fluoreszcenciás jel alapján méri. A modulok egyes biológiai vagy kémiai komponensek meghatározását azok közvetlen (direkt) fluoreszcenciája alapján, másokét valamilyen fluoreszcens festékanyag bekapcsolásával közvetett (indirekt) fluoreszcencia mérésével teszik lehetővé. Így a műszermodulok így az algasűrűségről illetve a szervesszéntartalomról, valamint

egyes szerves mikroszennyezők mennyiségi jelenlétéről biztosítanak mérési adatokat a vizsgált víztestben, az utóbbi célvegyületeket részint a direkt fluoreszcencia mérésével, részint immunfluoreszcenciás mérési módszer alkalmazásával detektálva.
Kulcsszavak: *fluoreszcencia, vízanalitika, mikroszennyezők, zearalenone, glyphosate*
JEL kód: *Q25*

Introduction

Water management is of increasing importance due to decreasing availability of drinking water and increasing water quality problems, and quality assessment is an essential task in the control of water resources.

Fluorescence, a stunning interaction between light and matter, a special form of luminescence, is the phenomenon when a given substance emits light upon previous absorption of (excitation by) light or other electromagnetic radiation. The emitted light is, in most cases, of a longer wavelength (i.e., lower energy) than the absorbed radiation (Figure 1). Nonetheless, it is also possible that emission is at a lower wavelength (e.g., in the case of two-photon absorption) or at the same wavelength (resonance fluorescence) as the irradiation. The process of fluorescence generation proceeds in three major steps: (a) excitation of a susceptible molecule by an incoming photon (an event occurring in femtoseconds); (b) vibrational relaxation of excited state electrons to the lowest energy level (occurring in picoseconds); and (c) emission of a longer wavelength photon as the molecule returns to ground state (occurring in nanoseconds). Fluorescence is particularly spectacular, when the absorbed radiation is in the ultraviolet spectrum (non-visible), but the emitted light is in visible.

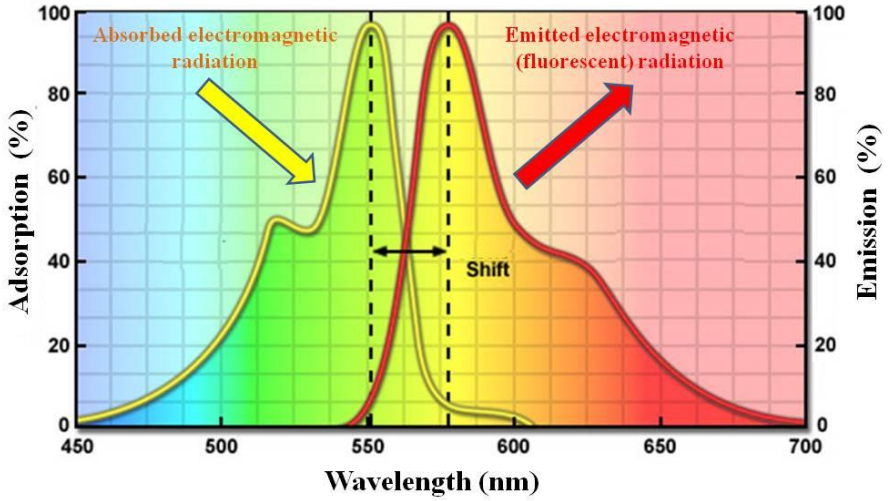

Figure 1. Fluorophore absorption and emission profiles.
The energy of the absorbed electromagnetic radiation is transformed to higher wavelengths and lower energy levels in form of the emitted fluorescence. (Note: Intensities of the incoming and emanated radiations have been normalized. In reality the process is accompanied by dispersal of energy.) (Based on CARL ZEISS CO., 2022).

Due to its unique optical features, tremendously sensitive emission profiles, spatial resolution, and high specificity, fluorescence gain practical applications in a wide range of specialties e.g., fluorescent lamps, analytical chemistry (fluorescence spectroscopy), sensorics (chemical sensors), mineralogy, gemology, as well as fluorescent labeling – use of fluorescent dyes to label various substances, including proteins, receptors and other biochemicals to use

them in biological detection (WEHRY, 1981; BRIGHT, 1988; LAKOWICZ, 2006). Interest in fluorimetry is increasing also in water quality research (HENDERSON et al., 2009) as the non-invasive optical sampling combined with the high detectability of fluorescence signals provide an efficient means of tracing organic contaminations in water bodies. Upon excitation with an energy source, a typical river water sample will display a range of fluorescent emissions, which include protein-like (e.g. tryptophan, tyrosine) and fulvic/humic acid-like fluorescence. These emissions occur at distinct wavelengths and are therefore readily identifiable in emission spectra.

Project AQUAFLUOSENSE

To utilize the advantages of fluorescence spectroscopy in water analysis an innovative development project was launched in 2017 in the scope of the National Competitiveness and Excellence Program (NVKP_16) financed by the National Research, Development and Innovation Fund. Project AQUAFLUOSENSE (NVKP_16-1-2016-0049) combined complementing expertise of several leading research and development institutions and business partners in Hungary (Aquafluosense, 2022): the fundamentals and design of optical systems at the Department of Atomic Physics, Budapest University of Technology and Economics, the concepts and development of instrumental and immuodetection-based methods in analytical chemistry at the Agro-Environmental Research Institute and the Food Science Research Institute at the National Agricultural Research and Innovation Centre (currently belonging to the Institute of Environmental Science and to the Institute of Food Science and Technology at the Hungarian University of Agriculture and Life Sciences, respectively), detailed expertise on aqueous contaminants and algal populations in water bodies and their reservoirs at the Danube Research Institute of the Ecological Research Center (belonging then to the Hungarian Academy of Sciences), as well as practical knowledge on the development of optical instruments and commercial immunoassay kits at business firms Optimal Optik Ltd. and the Institute of Isotopes Co., respectively.

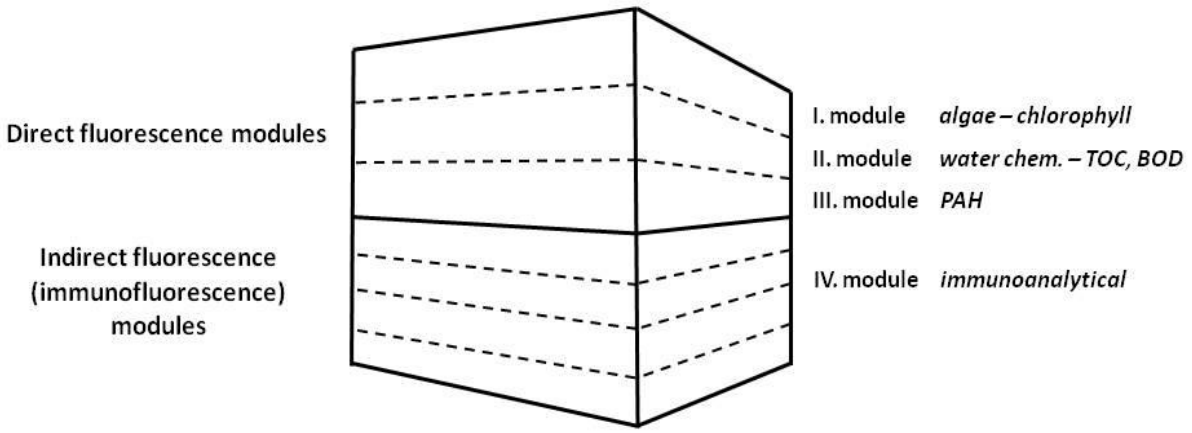

Figure 2. Schematic setup of the modules of the fluorometer prototypes.
Certain substances e.g., chlorophyll (algal density), tryptophan (correlating with total organic carbon, TOC and biological oxygen demand, BOD), and polycyclic aromatic hydrocarbons (PAHs) are detected by their direct fluorescence. Others are determined in immunoassay protocols using fluorescent labels.

In the scope of the project, completed in 2021, the design and construction of a prototype of an electron excitation fluorescence-based analytical instrument family has been carried out for water quality measurement applications. The objective of the project was to develop a new water analysis system for natural and artificial waters, allowing complex, systematic and for main parameters in situ assessment and monitoring of water quality, by developing a modular instrument family that can be individually configured for target tasks at each monitoring point. Within the instrument family different modules allow for the determination of key water parameters. A common optical characteristic of these modules is that they measure the target parameter on the basis of an induced (excitation) fluorescence signal generated in the test sample. The modules allow the determination of individual biological or chemical components based either on measuring their fluorescence directly (direct fluorescence) or by relying on detection of the fluorescence of a coupled dye (indirect fluorescence) (Figure 2).

As seen from the instrument setup, the instrument modules provide experimental data on the algal density and the total organic carbon content, as well as on the presence of certain organic micropollutants in the given water body studied, the latter target analytes detected by direct fluorescence measurement or by an immunofluorescence measurement modality. The optical systems of the instrument and given modules are summarized below according to their corresponding target analytes.

The optical system of the instrument

A key element in the optical system development is the light source used to illuminate the sample to induce excitation fluorescence. The samples were illuminated by an LED light source with a 520 to 535 nm minimum to maximum dominant wavelength range, by a Xenon flash lamp or by a 10-25 mW laser diode at excitation wavelengths of 635 or 637 nm. The emission was detected by a dichroic beam path with silicon photodiodes having a large active area. The necessary high spectral blocking and contrast were achieved by a combination of dichroic and bandpass filters. It was also possible to capture the entire fluorescence spectrum by a spectrophotometric detector applied, proving to be sufficient to distinguish between different fluorescence components. Detection at fixed wavelengths is much more cost-efficient, although its spectral resolution is limited by the optical bandpass filters available. In order to achieve high sensitivity, signal averaging was accompanied with active noise reduction (synchronous detection of a low photon count, modulated probe light separated from the actinic light). Since fluorescence emission is orders of magnitude lower than the excitation, relative sensitivity was in the range of 10^{-4} to 10^{-3}. It was necessary either to use sufficient excitation intensity (I_{exc}), or to increase the passband width of the filter ($\Delta\lambda$). As sample response is highly dependent on the excitation characteristics, the excitation intensity dose ($I_{exc}\Delta t$) had also to be considered (BARÓCSI et al., 2018; CSŐSZ et al., 2019).

Instrument prototypes in most cases were equipped with a sample holder devised to comply with the 96-well enzyme-linked immunosorbent assay (ELISA) microplate format, with a mechanical or motorized stepping device to carry out individual measurements in the individual wells of the microplate (Figure 3). Alternatively, in other cases, fluorescence measurements were carried out in the standard cuvette format. The instrument modules have also been installed in a mobile laboratory motor vehicle that allows *in situ* analytical determinations at the sampling sites (Figure 3).

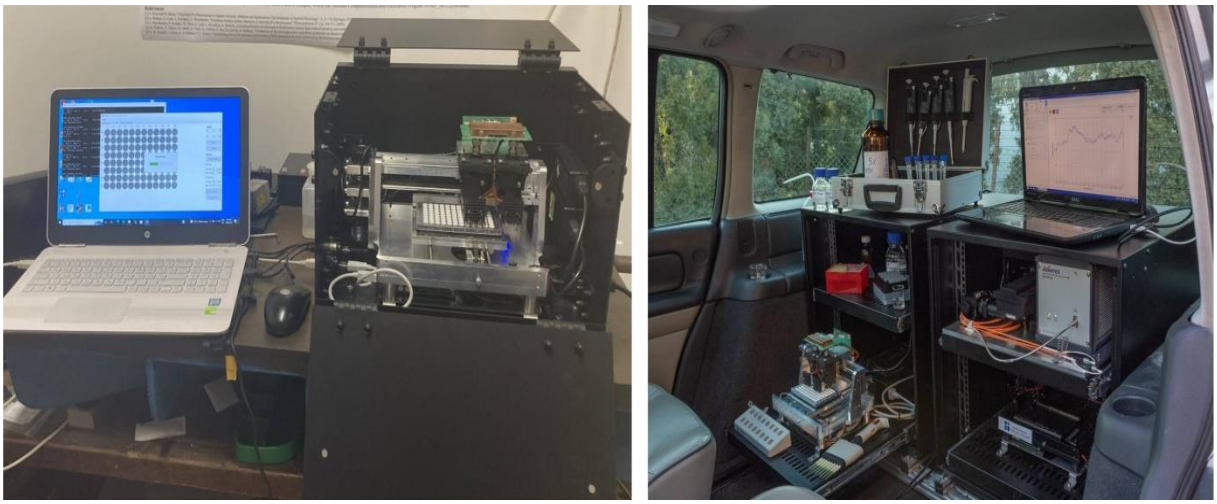

Figure 3. Schematic setup of the modules of the fluorometer prototypes.
Photographs of the algal density fluorometer module (*left*) and the AQUAFLUOSENSE instrument modules during on-site operation in a laboratory motor vehicle (*right*).

In the modular setup the instruments made several parallel water quality parameter determinations possible. These application possibilities have been presented in international and domestic scientific events (SZÉKÁCS, 2019; ADÁNYI et al., 2019) and have also been discussed for practical utilization with the Hungarian National Directorate General for Disaster Management, Ministry of the Interior. (For description and references of individual applications see in detail below.)

Direct fluorescence module for the detection of algal density

Induced fluorescence can be used to measure chlorophyll and the method of fluorescence-based chlorophyll measurement was developed as early as in the 1970s (KIEFER, 1973) and different pigment composition in different algal groups allowed distinction among these main groups based on their fluorescence characteristics (YENTSCH – YENTSCH, 1979; YENTSCH – PHINNEY, 1985; COURTECUISSE et al., 2022). Thus, fluorometers are available for chlorophyll content estimation (BEUTLER et al., 2002), although their efficacy in differentiation among different algal compositions is disputed (KAHLERT – MCKIE, 2014).

Based on the emission fluorescence spectra of species of different algal phylae, excitation wavelengths of 470 nm and 630 nm were selected for the fluorometer prototype to detect algal density in aqueous samples. At these excitation wavelengths, emission peaks were detected at 690 and 660 nm, respectively. The detection wavelengths were selected taking into account the emission spectra, as well as other important electrotechnical and optical aspects required by the module development (LÁZÁR et al., 2019, 2020). Results were validated by other methods measuring algal density: (i) optical density at 750 nm, (ii) counting in a Bürker-chamber and (iii) chlorophyll measurement with the organic solvent extraction method. Thus, the instrument module prototype provided validated, good quality results for the quantification of algal biomass. Both the limit of detection (LOD, 2.22-3.70*10^3 cells/ml) and limit of quantification (LOQ, 2.65-6.10*10^3 cells/ml) indicated a substantial improvement during the development process, and the prototype is considered sensitive and efficient in regard to these parameters.

Direct fluorescence module for the detection of total organic carbon

Reference standard laboratory methods are available for determination of water quality parameters, including conductivity, pH, temperature, turbidity, as well as chemical oxygen

demand (COD), biological oxygen demand (BOD) and total organic content (TOC). Fluorescence spectroscopy has been intensively applied in the recent decades in the analysis of dissolved organic matter (DOM), in hope to extend analytical capacities with *in situ* methods. Such determinations are possible, because some DOM fractions are fluorescent, and these fractions can be further classified into tryptophan-like, tyrosine-like and humic acid-like substances. Tryptophan in water is originated from microbial processes, and its concentration (the intensity of tryptophan fluorescence) has been indicated to correlate with the biochemical oxygen demand (BOD). Consequently, through correlating with tryptophan concentration, the fluorescence signal of tryptophan can provide a means to monitor BOD (KHAMIS – STEVENS, 2013).

In the prototype instrument module developed a standard quartz cuvette was excited with a Xenon flash lamp combined with an optical bandpass filter. The fluorescence was measured with a high-sensitivity, thermoelectric-cooled charge-coupled device (CCD) fiber-optic spectrometer or a photomultiplier tube combined with a set of optical interference filter. The fluorescence of the solutions tryptophan and tyrosine excited at 311 nm, 335 nm and 377 nm, and emitted fluorescence at 256 and 276 nm, respectively. Spectra of tyrosine, tryptophan, humic acid, COD standard solution were collected at different concentrations to obtain a range of measurement. Fluorescence intensity obtained with given humic acid samples interestingly showed no monotonous concentration dependence. At the maximal absorbance wavelength for a COD standard solution (339 nm), fluorescence intensity was correlated with TOC, and a moderate to good correlation (R^2=0.83) was found. Thus, the measured fluorescence intensity provided information on TOC with a good accuracy. A "fingerprint map" based on the fluorescence spectra of standard solutions was established from chemometric processing of the signals obtained from the results at corresponding characteristic emission wavelengths. Both the newly developed module and the analytical procedure were validated with reference measurements. The aim with this was to identify tyrosine-, tryptophan- and humic acid-like regions in the characteristic emission wavelength parameter space. Such mapping was found to be reproducibly applicable in surface water samples, although a slight dilution (5-10-fold) was occasionally needed to avoid matrix effects. With this method facilitated by chemometry differentiation between COD-, tryptophan- and humic acid-like could be demonstrated (KÓNYA et al., 2019). In contrast, waste water has unfortunately been found to be an overly complex matrix with high variability among individual samples of different origins, therefore these samples required a 50-100-fold dilution to avoid matrix effects, which limits method applicability in waste water.

Direct fluorescence module for the detection of polycyclic aromatic hydrocarbons

PAH substances consisting of two or more condensed aromatic rings are ubiquitous environmental pollutants. Many PAHs have been proven to exert toxicity, carcinogenicity and mutagenicity to mammals including humans. Therefore, European Parliament and Council Decision 2455/2001/EC (EUROPEAN COUNCIL, 2001) enlists certain PAH compounds as priority pollutants among substances or groups of substances that have been prioritized for action at Community level. Priority pollutant PAHs include anthracene, benzo[a]pyrene, benzo[g,h,i]perylene, benzo[k]fluoranthrene, benzo[b]fluoranthrene and indeno[1,2,3-cd]pyrene, and Directive 2008/105/EC (EUROPEAN COUNCIL, 2008) sets strict environmental quality standards (EQS) to regulate the annual average value (AA) and the maximum allowable value (MAC) of priority substances in surface waters.

A fluorometer module was developed for rapid determination of the most important PAHs by using direct fluorescence techniques. The instrument prototype utilized a high power LED lamp combined with an optical bandpass filter (257 nm), and fluorescence was measured with a high-sensitivity, thermoelectric cooled CCD array fiber-optic spectrometer. Similarly to the COD

module, emission spectral characteristics, calibration curves, LODs and a „fingerprint map" were established for priority PAHs from the chemometric processing of signals from corresponding characteristic emission wavelengths, and the module and the analytical procedure were validated based on reference high-performance liquid chromatography (HPLC) measurements. Wastewater samples of different origin and wastewater spiked with PAHs (pyrene, anthracene, chrysene, and phenanthrene) were analyzed, and results confirmed detection of PAH concentrations at about the emergency level (BERKI et al., 2020).

Immunofluorescence modules for the detection of organic microcontaminants

Fluorescence detection may offer utility in monitoring compounds that lack any fluorophores if the use of a fluorescent dye is possible. The only principal issue is specificity (specific placement of the fluorescent dye), and the protocol of enzyme-linked fluorescent immunoassay (ELFIA) methods provide a solution for that. The source of specificity in immunoassay is rooted in the biochemical features of natural antibodies raised against haptenic derivatives of the target analytes (TIJSSEN, 1985). Such ELFIA systems have been developed for the detection of several environmental xenobiotics, including mycotoxin zearalenone (ZON) (Gémes et al., 2020, 2021) and herbicide active ingredient glyphosate (TAKÁCS et al., 2022). The immunofluorescence module prototype used an LED light source (a 520 to 535 nm minimum to maximum dominant wavelength range, and the emission was detected by a dichroic beam path with photodiodes. Dichroic (edge: 562 nm) and bandpass optical filters (excitation: peak: 531 nm, width: 40 nm; emission: peak: 593 nm, width: 40 nm) were applied. Upon a two-stage amplification, the photodetector signal was fed to an analog-to-digital converter yielding 4095 resolvable relative fluorescence units. A 96-well microplate-based immunoassay format was used. The microplate-based fluorescence instrument has been demonstrated to be capable of detecting ZON and glyphosate in the concentration range of 0.09–400 ng/mL and 0.09–100 ng/mL, respectively. The sensitivity and accuracy of the analytical method was validated by HPLC measurements, and ZON additionally with total internal reflection ellipsometry (NABOK et al., 2021), and the same immunoreagents were also applied in an immunofluorescent capillary sensor for the detection of ZON (MAJER-BARANYI et al., 2022). An important feature of these ELFIA methods is their efficacy in combined application possibility. The specificity range of the immunofluorescence module can be and is being further expanded to other organic micropollutants with the use of novel antibodies as they become available for detection.

Conclusion

A portable, modular structured instrument was developed for rapid determination of the most important water quality parameters by using direct fluorescence techniques. The main advantage of the fluorometer module prototypes is their combined *in situ* applicability for determination of various important water quality parameters detectable by induced fluorimerty e.g., TOC, algal density or the level of micropollutants detectable by immunofluorimetry. In addition, as pointed out, the immunofluorescence module can readily be widened to other target analytes. The project was completed in 2021, but results spin-off and become published to date, and the fluorometer modules are likely to be used in various practical applications in the near future.

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