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Real-time monitoring of bacterial and organic pollution in a water stream by fluorescence depletion spectroscopy

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Abstract We demonstrate an approach for a real-time, consumable-free optical system operating on a liquid jet which can be easily derived from the water distribution infrastructure. We apply a pump-probe scheme based on the acquisition and nanosecond manipulation of UV-excited fluorescence to increase the selective identification of bacterial against organic pollutants in water.

1 Introduction

The supply of high-quality drinking water is a major safety and health concern. Pollution of drinking water can be associated with two major families of contaminants: microbial and chemical (arsenic, nitrate, and heavy metals) [1]. Polychromatic hydrocarbons (PAHs), whose origin is both natural (forest fires) and anthropogenic (fossil fuels combustion, waste incineration, etc.) [2], are a particularly insidious group of chemical organic contaminants, and in fact, many of them are enlisted as priority pollutants by the European Commission [3]. Although typically associated with atmospheric pollution, they can be introduced in potable water infrastructure via water treatment plants and rainwater collecting basins. The identification of PAHs in water is normally based on liquid and gas chromatography combined with mass spectrometry or optical detection (absorption and fluorescence) in the ultraviolet (UV). As for biological pathogens, public water analysis is performed following standardized ISO methods largely based on surface plating and membrane filter techniques [4, 5]. Alternatively, techniques based on multiple-tube fermentation [4] or titration of adenosine triphosphate (ATP) are accepted for their quantification [6]. All these approaches are associated with relative long incubation times necessary for performing the final assessments: a major limiting factor for both sampling volume and sampling frequency. Interestingly, it was demonstrated already in 1991 that even for water meeting microbiological standards, 35% of gastrointestinal diseases in the Montreal area were water-related and preventable [7]. These figures have unfortunately not changed substantially after 25 years [8]. For these reasons, new quality control solutions to complement existing ones are desirable. For instance, flow-based methods based on FACS (fluorescence activated cell sorting) technology are emerging thanks to their ability to increase testing rapidity and monitoring directly water streams within the water infrastructure [9]. To date, all FACS approaches are primarily addressing microbial pollution and they rely on a preliminary staining of water samples by molecular fluorescent probes targeting DNA, RNA, or bacterial species by antibody recognition. In this respect, they require non-negligible quantity of chemical consumables and also frequent interventions at the detection site by specialised operators, preventing fully autonomous and long-term operation. The picture above is going to be further modified by the determination by the public bodies of several countries to provide disinfectant-free water to avoid people exposure to chemical byproducts (e.g., chlorine, chlorine dioxide, chloramines, and also halogenated PAHs deriving from the chlorinization of PAH-polluted water [10]) and improve potable water taste. This paradigm change in water distribution may lead to several

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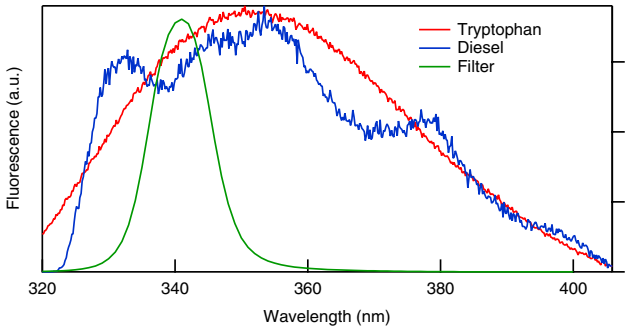

Fig. 1 Normalized UV-excited (266 nm) fluorescence spectra of tryptophan in DPBS and Diesel acquired using an imaging spectrometer equipped with an EM-CCD. The *green curve* represents the nominal transmission curve of the filter used in detection for this work

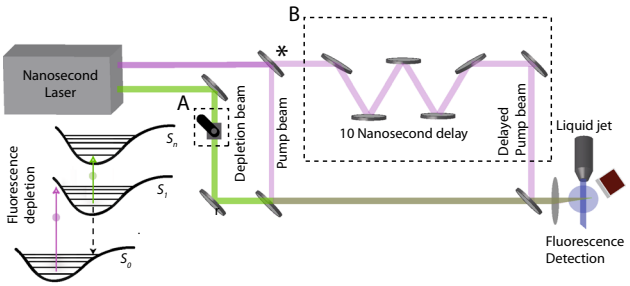

Fig. 2 Experimental set-up. **a** Shutter is used to generate pump + probe and pump only sequence. In this case, the optical element indicated by *asterisk* is a 100% reflective mirror. **b** As alternative configuration, 50% of the pump beam is retarded by a fixed delay line by several nanoseconds to interrogate the fluorescence response of the same sample volume. In this case, the shutter is not used and *asterisk* represents a 50% beam splitter. *The fluorescence depletion scheme* on the *left* shows the molecular levels involved in the process. S_0 is the ground state; S_1 the first excited state. Fluorescence stems from the $S_1 \rightarrow S_0$ radiative transition. S_n is a higher lying state which relaxes non-radiatively. If the electronic population is promoted from S_1 to S_n by the depletion (*green*) beam, a decrease in the intensity of the fluorescence signal is observed

infection-related health issues and could be made possible only if supported by improved sensor technology with real-time read-out [11].

Although capable of real-time operations, optical detectors lack in specificity. For instance, the UV-excited fluorescence from organic compounds like PAHs cannot be easily distinguished from that stemming from biological species [12]. As an example, in Fig. 1, we show the strong overlap of UV-excited fluorescence from tryptophan in DPBS (Dulbecco's Phosphate-Buffered Saline) and Diesel fuel. Our group demonstrated that this selectivity issue can be overcome by pump-probe depletion spectroscopy [13, 14]. This approach can be summarized as follows (see scheme in Fig. 2): a first UV pulse exciting the molecule from the electronic ground state (S_0) to a fluorescing state S_1 is followed by a visible pulse interacting with the system before fluorescence is emitted. The existence of S_n states that are accessible from S_1 upon absorption of a probe photon entails a decrease in the $S_1 \rightarrow S_0$ fluorescence emission, provided that pulses duration and temporal delay are shorter than the fluorescence lifetime, which spans from a few (amino acids) to hundreds (PAHs) nanoseconds. We observed that the presence of accessible S_n states, and hence, depletion is common for biological fluorophores like tryptophan but not for PAHs. The advantage of this approach is that, although it involves subtle energetic and dynamical properties of molecules to increase selectivity, the experimental observable is simply fluorescence intensity, I, with clear benefits in terms of sensitivity and ease of implementation. Practically, one has to compute the depletion percentage D, $D = \frac{I_{\text{undep}} - I_{\text{dep}}}{I_{\text{undep}}} \times 100$, where the subscript labels indicate that if the fluorescence intensity is acquired in the presence (dep) or absence (undep) of the probe pulse.

2 Experimental set-up

For this demonstration, we used a nanosecond pulsed laser (Big Sky, Quantel, France) equipped with a nonlinear doubling and tripling frequency generation unit. The laser delivers probe pulses at 532 nm and pump pulses at 266 nm at 10 Hz repetition rate from two separate output ports. As indicated in the dashed box *A* in Fig. 2, a mechanical shutter is set on the probe beam before recombination with the pump beam to select the pulse sequence type (pump + probe or pump only). The beams are finally focused using a $f = 150$ mm lens onto a liquid jet generated by a sapphire nozzle (Kyburz-Sapphire, Switzerland). The sample is circulated by a peristaltic pump (Idex Health and Science, USA) with a net flow of 5 mL/s and a flow speed $v = 1$ m/s. Fluorescence is collected by a $f = 3,5$ cm, 1 inch diameter lens, spectrally selected by two equal band-pass interference filters (FB340-10, Thorlabs—see transmission curve in Fig. 1), refocused by a $f = 5$ cm onto the aperture of a pinhole in a confocal-like arrangement to increase spatial selectivity, and, finally, acquired by a photomultiplier tube (Hamamatsu H7826, Japan). For some specific measurements, the flow system was replaced by a static quartz spectroscopic cell with 1 cm optical path. This choice was dictated primarily to avoid flowing toxic solutions (e.g. fluorene), and then extended to safe samples to be able to compare their response under equal experimental conditions.

3 Results

For assessing the approach, we first measured UV-induced fluorescence intensity and depletion percentage for solutions at the same molar concentrations (1 mmol/L) of tryptophan (TRP, in DPBS) and fluorene (in ethanol) chosen here as molecular representatives of fluorescing biological molecules and polycyclic aromatic hydrocarbon (PAHs), respectively. Note that the fluorescence intensities and spectra are similar for both species (TRP, PAHs) preventing their discrimination based simply on these observables. In addition, fluorene differs from most of PAHs, as it is characterized by a short fluorescence lifetime similar to that of TRP [15]. Therefore, lifetime cannot be applied as a discrimination *criterion*. Finally, to get more insight into the final use of this spectroscopic approach, we measured also samples containing *Enterococcus Faecium*, a bacterial strain often found in contaminated water, and Diesel representing fossil fuel pollution.

The results reported in Fig. 3 show the different behaviour in terms of fluorescence depletion of biological (TRP and bacteria) and organic (Diesel and fluorene) compounds and the effect of green probe pulse intensity. Each panel contains two histograms obtained from depleted (blue) and undepleted (red) fluorescence signals from 30 successive laser shots. The different rows correspond to various green laser intensities ranging from 6.7×10^6 to 251×10^6 W/cm^2. These measurements, conducted in a static cell and not in flow, indicate that depletion is associated exclusively with biological samples and not present for organic PAHs. The depletion percentage for the former category grows with increasing green laser intensity, attaining ≥40% for 251×10^6 W/cm^2, while for PAH, the parameter D remains always negligible.

Based on this outcome evidencing the possibility of identification of microbial pollution against waterborne organic PAHs, we further assessed the sensitivity of the system on a bacterial sample circulating in the liquid jet, to mimic real-time monitoring of a water stream. The results in Fig. 4 are obtained measuring absolute fluorescence and depleted fluorescence intensities on water samples from the public infrastructure with the addition of known concentrations of *Enterococcus Faecium*. Concentrations are expressed as the number of bacteria in the focal volume, corresponding to $V = 2\times10^{-4}$ mL. For this series, the green laser intensity was set to 102×10^6 W/cm^2. Each point is calculated as the average of 100 laser shots. Fluorescence

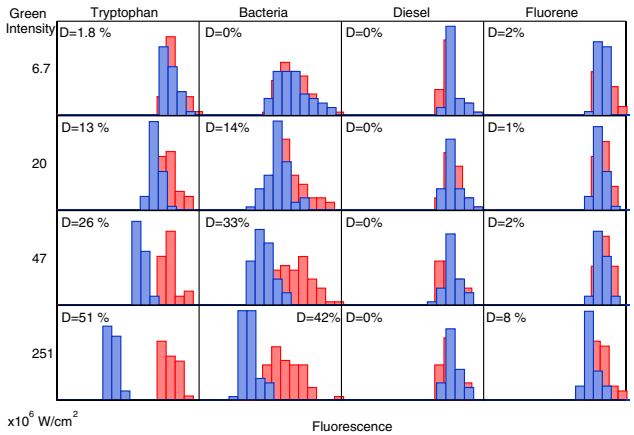

Fig. 3 Histograms for depleted (*blue*) and undepleted (*red*) fluorescence from different compounds (*columns*) at different *green* laser intensities (*rows*). The depletion parameter D is reported for each case. The *horizontal axes* always range between 0 and the maximum fluorescence intensity value

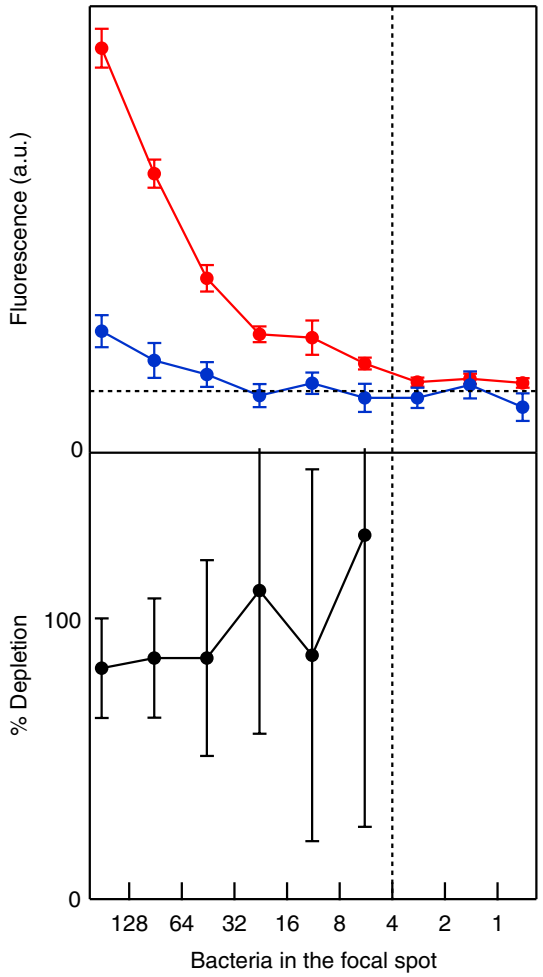

Fig. 4 *Upper plot.* Depleted (*red trace*) and undepleted (*blue trace*) fluorescence intensity of a water solution containing a variable concentration of *Enterococcus Faecium* bacteria. The *horizontal dotted line* indicates the signal offset. *Lower plot.* Fluorescence depletion percentage, D, calculated for the traces in the *upper plot*. The *vertical line* corresponds to the detection limit

intensity decreases with bacterial concentration towards an asymptotic value corresponding to roughly the intensity obtained for four bacteria in the focal spot. The limit of detection of the device (vertical dotted line) is ultimately bounded by this signal offset (horizontal line), which was determined in an uncontaminated water sample. By spectrally resolving the emission using an imaging spectrometer equipped with an EM-CCD, we could unambiguously attributed its origin to a Raman water peak occurring at 292 nm for 266 nm excitation. We assume that a better spectral selection (different spectral filters and detection geometry) would strongly benefit to the sensitivity of the set-up. In terms of selectivity, we observe that depleted fluorescence intensity is systematically much lower than undepleted one in line with the results of Fig. 3 for biological species. In the lower panel, we show the evolution of D parameter as a function of concentration. Here, for D calculation, $I_{\text{dep, undep}}$ signals are corrected by their respective offsets, experimentally measured on a pure water solution. Depletion starts at approximately 80% and stays constant (within the error bars) for all the measurements, consistently with the homogeneous nature of the samples tested. Note that the higher D values obtained here for bacteria with respect to those in Fig. 3 originate from the offset determination and removal procedure.

4 Strategies for improving sensitivity

Our spectroscopy scheme is highly selective for discriminating biological against organic water pollutants and sensitive enough for detecting a few bacteria per focal spot. However, the present figures are not adapted for detecting contaminations within the concentration thresholds used for assessing drinking water by public infrastructures. In fact, our device sensitivity corresponds to 3×10^6 c.f.u./100 mL, where c.f.u. stands for colony forming units, i.e., viable bacteria. Murphy et al. define the upper limit of microbial concentration for qualifying a water source as *lightly impacted* to ≤10 cfu/100 mL [8].

Major improvements are, therefore, necessary to compete with state-of-the-art bioassays in terms of absolute sensitivity. We identify two independent routes to obtain better performances. The first one addresses the issue of sampled volume. In fact, the low repetition rate of the laser in connection with the arrangement labelled A in Fig. 1 implies that depleted and undepleted signals are associated with very different sample regions. This can be particularly problematic for applying the depletion approach for the detection of low concentrated pollutants, i.e., in heterogeneous samples. This issue can be circumvented using a higher repetition rate laser and implementing the arrangement B, which has already been proven successful in an atmospheric monitoring device based on a kHz laser source [14]. In this case, the pump beam is split in two arms by a 50% beam splitter. One of the replica is retarded by approximately 10 ns by a 3 m delay line and then re-collimated with the pump + probe beam by a second dichroic mirror. Note that in this configuration, the shutter is not present. The delay among pump alone and the pump + probe pulse pair is short enough with respect to the flow velocity v that the same volume of the sample is interrogated but sufficiently long to avoid any temporal overlap among the two fluorescence responses.

As for the second upgrade, we remark that with the present configuration based on a laser beam orthogonal to the stream, only a very limited sample volume is probed and the available laser photons are barely absorbed in the thin water section. For improving these figures, one can opt for a longitudinal (with the laser beam parallel to the stream in a defined interaction region) or a folded (with several laser reflections on the inner walls of the water channel) arrangement with a collimated laser beam. Assuming that the focal intensity used in this work is preserved along the propagation (which is reasonable due to the low concentrations of absorbing molecules), and keeping the sensitivity value obtained here, we calculate that the threshold of 10 c.f.u./100 mL could be reached for a cylindrical interaction volume of 1 cm diameter and 76 cm length using 10 ns, 2J pulses, which can be provided by commercially available Nd:YAG laser sources. Note that this calculation does not account for any additional sensitivity gain obtained by introducing a volumetric integration of fluorescence instead of the simple collection by a low NA lens as shown here.

5 Conclusions

In conclusion, we have demonstrated an approach for setting-up a real-time, portable, and totally consumable-free optical system operating on a liquid jet which can be easily derived from the water distribution infrastructure. A single pulsed laser is used to set up a straightforward pump-probe scheme for real-time monitoring of water streams allowing the selective identification of bacterial against organic pollutants based on the acquisition and nanosecond manipulation of UV-excited fluorescence. We also discuss technological improvements of the set-up for gaining sensitivity comparable with that of state-of-the-art approaches and leading to a major increase in the sample volume probed.

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