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ORIGINAL PAPER

Bacteria Spectra Obtained by Laser Induced Fluorescence

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Abstract Laser-induced fluorescence technique (LIF) is described for getting bacteria spectra in the liquid phase. An excimer laser pumping a dye-laser and a doubly frequency laser have been used, exciting the bacteria to 290 nm, and using a monochromator, 600 lines/mm grating, with a CCD to measure and obtain the fluorescence spectra. In this study, a laser induced fluorescence system to measure certain bioaerosols (bacteria) was optimised. Finally, a small bacteria fluorescence spectra collection is presented.

Keywords Bacteria · Bioaerosol · Laser induced fluorescence

Introduction

Bioaerosols are aerosols of biological origin, and can be viruses, bacteria, fungi and pollen [1]. However, they can be treated as a special category of aerosols because their biological properties can affect the health of humans, animals and plants. This makes the development of analytical techniques particularly interesting, although difficult with limitations in sample collection and manipulation.

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There are hundreds of sources of microorganisms and is important to research techniques that enable rapid measurement of their composition and concentration, particularly in order to study their effects for health [2].

Bioaerosols present a special challenge in real-time compounds characterization. Spurny [3] made a compilation of different bioaerosols detection methods. These included microscope-based morphological evaluation of organisms, culture techniques, chromatography, mass spectrometry, luminescence, infrared spectroscopy and Raman and certain other biosensors (inmunobiological techniques using electrochemistry, radioactive label . . .).

In the last decade, some studies have been published on the real-time characterization of individual particles. In the specific case of atmospheric aerosols, the most used technique employs an ionisation laser connected to a time-of-flight mass spectrometer [4]. Generally, the most important shortcoming of this technique is the difficulty to obtain quantitative data, since the complete chemical composition of the particle cannot be determined until it has completely vaporised, atomised and ionised. Nevertheless, this method offers a huge quantity of information and is one of few capable of providing information in real time.

For particles with luminescent properties, which it is the case of the most organic aerosols and bioaerosols (e.g., aromatic polycyclic hydrocarbons), laser-induced fluorescence has been used as method for particles characterization [5]. The simple presence of fluorescence can be used to distinguish between biological and non- biological particles and the spectra of individual particles can yield much more detailed information. Thus, for example, Chen et al. [6] used an aerosol generator, separated from a nebuliser, and well as filters, an impactor and a system for controlling particle size to obtain reproducibility in the size of bioaerosols and later

the fluorescence spectra of *Escherichia Coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus thuringiensis*.

Pan et al. [7] identified four allergy-producing substances (e.g. oat pollen or mushroom spores) using 266 nm as an excitation wavelength and a CCD as a detector. These authors also identified riboflavin and *Bacillus subtilis* spore particles. Nevertheless, the identification of specific bacteria (such as *Escherichia coli vs*. *Staphylococcus aureus*) is not easy since their spectra are very similar, as reported by Seaver et al. [8].

The general aim of this study was to perfect and optimise a system of laser- induced fluorescence for measuring certain bioaerosols working in the liquid phase. Concretely, a small bacteria fluorescence spectra collection is presented, which most of them have not described before in the bibliography. After optimizing this system, the next step will be to work on the gas phase, generating the bioaerosol in the laboratory.

Experimental

Instrumentation

The analytical chemistry laser laboratory in the University of La Rioja where the experiment was carried out has all the necessary safety equipment. The halogen gases were used in cupboards (Praxair España S.L., Madrid, Spain) and certified by continuous extraction.

A schematic set up of the system is shown in Fig. 1. An excimer laser XeF emitting at 351 nm pumped a dye laser with rhodamine 6G. The dye laser was coupled to a frequency doubler. The final laser radiation was directed at the sample using two mirrors and the fluorescence emission was collected with a concave mirror and focused with a lens towards the monochromator (grating of 600 lines/mm) and the detector (CCD). The CCD was programmed with Andor MCD software, version 2.62 I2C (Andor Technologies). Two cut-off filters were used to eliminate radiation below 295 nm and above 540 nm.

Lambda-Physik excimer laser, model Compex201, Lambda-Physik dye laser, model ScanMate 2, Lambda-Physik frequency doubler, model SHG-crystal BBOI (295–220 nm).

Two Newport mirrors (ref. 10D10AL.2) and one Newport concave mirror (ref. 10DC50AL.2). A Newport lens (ref. SBX022). Two cut-off filters, Coherent (ref. 35-5321-000) and Schott Glass (ref. 117303).

Monochromator Bentham Instruments, model TM300 (three gratings of 2400, 1200 and 600 lines/mm), detector CCD (Andor Technologies DV401-UV 256 × 1024 pixels).

Quartz cell (1 cm path length) Hellma101QS, quartz flow cell (4 mm path length) Hellma 176.050 QS and 75 μm inner diameter capillary Teknokroma TSU075375.

Masterflex 7518-10 peristaltic pump.

Two Optika microscope microscopes, 40x/0.65 and 60x/0.85.

Lambda-Physik (Göttingen, Germany). Newport (Irvine, CA, USA). Coherent (Dieburg, Germany). Schott Glass (Barcelona, Spain). Bentham Instruments (Berkshire, UK). Andor Technologies (Belfast, Northern Ireland). Hellma (Barcelona, Spain). Teknokroma (Barcelona, Spain). Masterflex belongs to Cole-Palmer Instrument Company (Vernon Hills, IL, USA). Optika microscopes (Ponteranica, Bg, Italy).

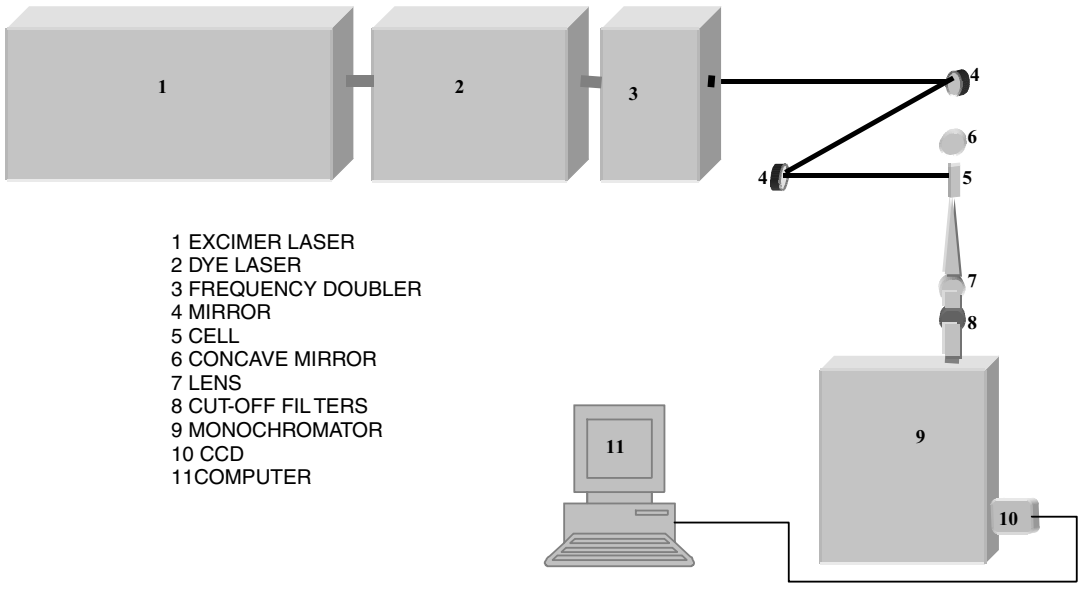


Fig. 1 Schematic set up of the laser induced fluorescence system

Reagents and biological material

Pure fluorescein and anthracene >99% were purchased from Fluka (Buchs SG, Switzerland), ethanol absolute from Panreac (Barcelona, Spain) and the pure water was obtained from a Milli-Q apparatus (Millipore, Bedford, MA, USA).

Bacteria were obtained from the Spanish Collection of Type Cultures. Lyophilised bacteria were hydrated and cultivated in Petri dishes with the appropriate culture medium, normally blood agar or milk agar. The plates were incubated at 37°C (Selecta, Barcelona, Spain) to induce bacteria growth. Then a platinum looped tube sterilised by fire was used to transfer the microorganisms to test tubes containing pure water previously sterilised in an autoclave (Autotester-G, Selecta, Barcelona, Spain). The concentration was expressed as colony-forming units per millilitre (cfu/mL) and determined by comparison with McFarland standards (Biomerieux España SA, Madrid, Spain).

All residue was sterilised in the autoclave before being discarded. The material that could not be introduced in the autoclave was sterilised with absolute ethanol.

Procedure

The following procedure was used to obtain the fluorescence spectra of the bioaerosols studied. The measurement conditions were adjusted and optimised: excimer laser (75 mJ, 5 Hz), dye laser (rhodamine 6G), frequency doubler with BBOI crystal, grating with 600 lines/mm and CCD (0.2 s as exposure time, 50 accumulations). Excitation radiation was selected at 290 nm and directed at the sample, which was in a conventional fluorescence quartz cell with 1-cm path length. As shown in Fig. 1, a lens and a concave mirror were used to collect and focus the fluorescence radiation. The signal was registered with the detector and the fluorescence spectrum obtained. Spectrum data in the ASCI file were exported to the Origin 6.0 programme for processing and drawing.

The sample solution was prepared as described in the previous section (reagents and biological material).

Results and discussion

Previous tests

The laboratory where the research experiment was carried out was only set up and assembled recently, so many tests had to be carried out to adjust and optimise the laser-induced fluorescence measuring system, from the installation of safety systems to the alignment of the laser radiation.

First, we had to select the compounds that were going to be used to optimise the system. The main drawback in this sense was the limitation of the system itself, which was imposed by the dye and by the crystal frequency doubler. The dye used was rhodamine 6G, which can be used at between 569 and 608 nm; the frequency doubler uses crystal with a range of between 295 and 205 nm. Therefore, we were able to measure compounds that, when excited at wavelengths of between 285 and 295 nm, are able to emit fluorescent radiation. Of the 14 compounds studied, anthracene and fluorescein were selected for the optimisation because they were the least toxic. These compounds are excited at 290 nm. Stability studies were performed to ensure these compounds were stable, even after they had been radiated with a laser several times.

Excimer laser energy and frequency were two parameters studied, and although it is logical that stronger signals are achieved at higher energy and frequency levels, we chose 75 mJ and 5 Hz, respectively, although 300 mJ and 10 Hz can be reached with this equipment.

The monochromator used had three diffraction gratings: 2400, 1200 and 600 lines/mm. The more lines, the better the resolution but the smaller the spectral range. Figure 2 shows the fluorescence spectrum of an anthracene dissolution with the three gratings. At the time, we chose the grating with 600 lines/mm because the resolution was not considered to be that important.

A CCD was used as a detector and the parameters evaluated were temperature, accumulation number and exposure time. As is known, reducing the temperature of the CCD increases the signal/noise ratio, as shown in Fig. 3. Thus, the anthracene spectrum could be appreciated better when working at 0°C. Figures 4 and 5 show how the fluorescence spectrum changed when exposure time or the number of accumulations was modified, respectively. We selected an exposure time of 0.2 s and 50 accumulations in order not to prolong the analysis time excessively.

System optimisation

Once the above mentioned conditions had been established, we optimised the rest of the system. To facilitate exposure, the system was divided into three parts: (a) excitation radiation; (b) collection of fluorescent radiation; and (c) sample vessel.

The aim was to achieve greater sensitivity in the collection of fluorescence spectra. Methods for achieving this improvement include the following:

- using sample vessels smaller than 3 mm, which is the entrance width of the CCD.
- placing a focussing lens between the measuring cell and the monochromator; one *f*/2 lens is normally used at a distance of 1 to 1, but microscope lenses can also be used.

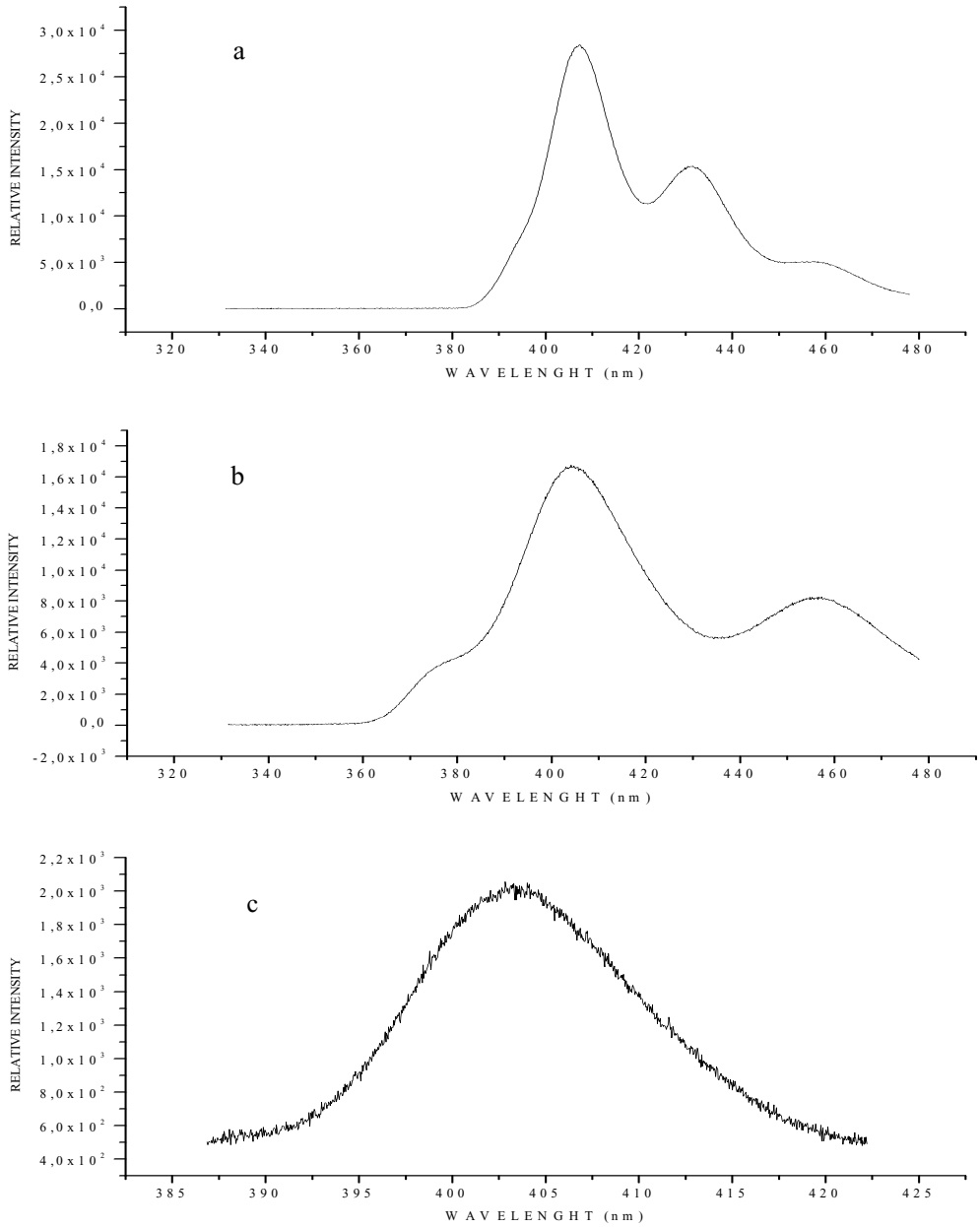


Fig. 2 Anthracene fluorescence spectra using different gratings: (a) 600 lines/mm, (b) 1200 lines/mm and (c) 2400 lines/mm

– collecting fluorescence emitted in all directions.
– using cutting filters to prevent Rayleight dispersion.

(a) Excitation radiation. Once laser radiation had been defined, the only two considerations taken into account before radiation reached the sample were as follows: the use of two mirrors to ensure radiation reaches the sample horizontally; and the use of a diaphragm to ensure radiation touches the selected sample points. This also avoids saturation problems if high concentrations are measured.
(b) Collection of fluorescent radiation. Two optical elements were used to improve the signal: a lens and a mirror. The lens was placed between the quartz cell and the monochromator and the mirror at 180° from the lens. Figure 6 shows the improvement in the fluorescence signal when only the lens was used. Although in theory it should have been placed in the 1-to-1 position, i.e. at the same distance from the monochromator as from the sample, the experimental data showed that this was not the case. An improvement was also observed when only the mirror was used in the system (Fig. 6). And combining the effect of both optical elements yielded the results shown in the same figure.

To prevent laser radiation reaching the detector (290, 580 nm), two cutting filters were used to eliminate radiation under 295 and above 540 nm.

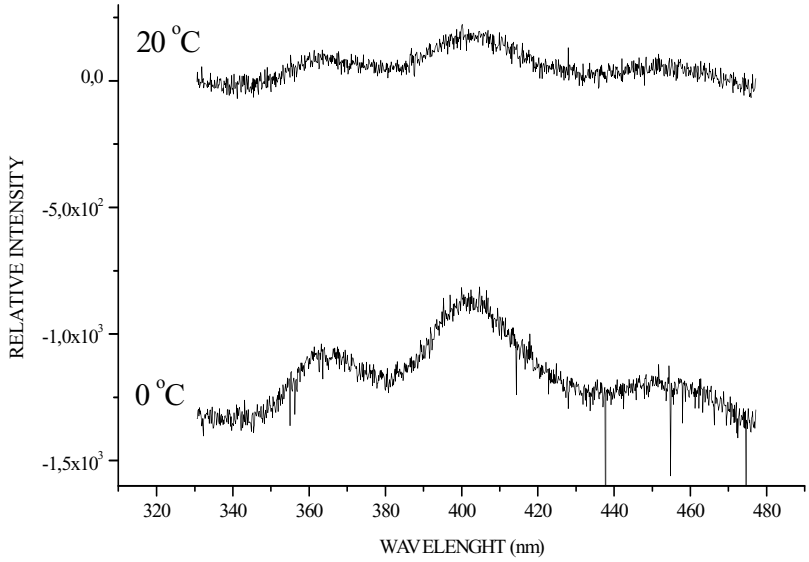


Fig. 3 Effect of the CCD temperature on the anthracene fluorescence spectrum

(c) Sample vessel. So far the trials were performed with a conventional 1-cm fluorescence cell. In this section we tested other vessels such as the continuous flow cuvette with an optical path length of 4 mm and the 75 μm inner diameter capillary. Microscope lenses were used with these two new vessels to focus excitation radiation on the sample. As shown in Fig. 7, the best signal was obtained with the conventional cuvette.

Bioaerosol fluorescence spectra

By using the procedure described in "Experimental" we obtained the fluorescence spectra of the following bacteria shown in Fig. 8:

Bacillus subtilis, *Niger*, CECT-38.
Enterococcus Faecalis, Z-397.
Enterococcus Faecium, Z-415.
Enterobacter Aerogenes, CECT-684.
Escherichia Coli, Co-15.
Lactobacillus Brevis, J-81.
Lactobacillus Plantarum, J-39.
Lactococcus Lactis.
Micrococcus Luteus, C-157.
Pediococcus, R-7.
Staphylococcus Epidermidis, Sa-41.

As can be seen, there were two peaks in all cases: one around 350 nm and another around 485 nm. However, these spectra can be useful for classifying bacteria into their corresponding group, bacilo, coco, enterococo, gram + or gram − , ... The next step will be to obtain more fluorescence spectra and to classify them using chemometric tools.

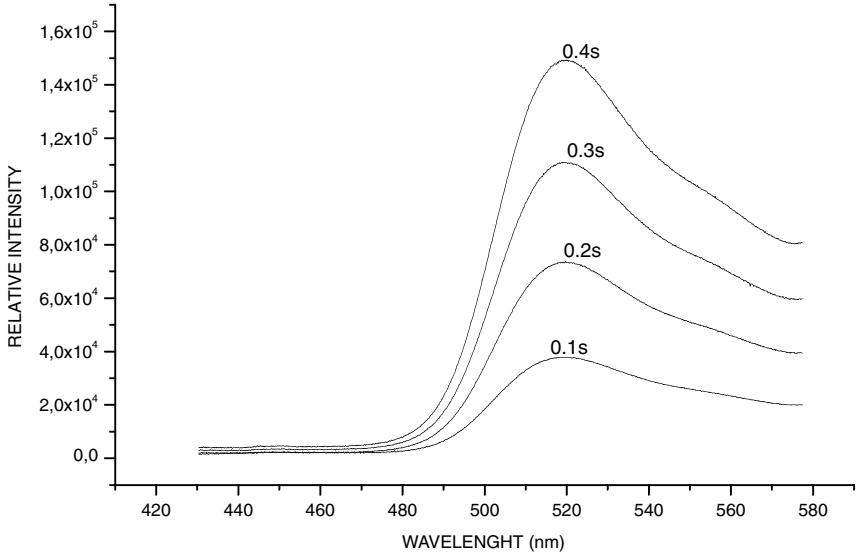


Fig. 4 Variation of the fluoresceine fluorescence spectrum using different exposition times on the CCD: 0.4, 0.3, 0.2 and 0.1 s

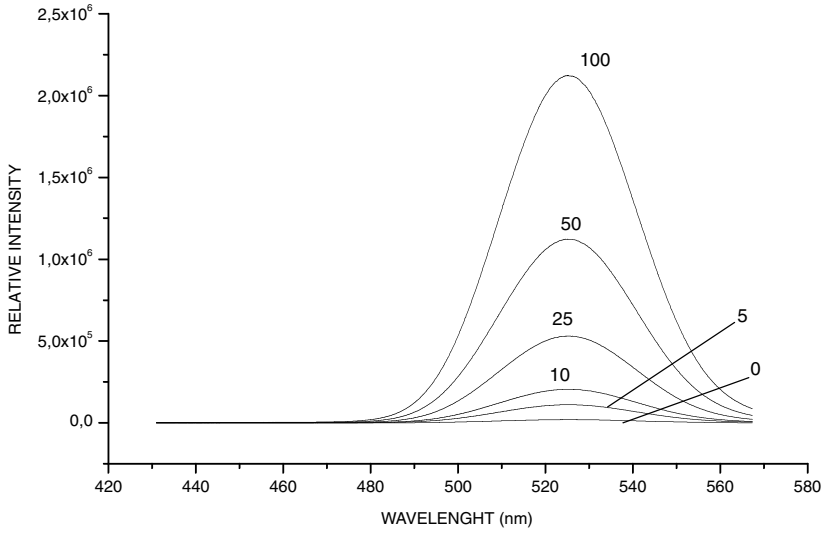


Fig. 5 Variation of the fluoresceine fluorescence spectrum using different accumulations number on the CCD: 100, 50, 25, 10, 5 and 0 accumulations

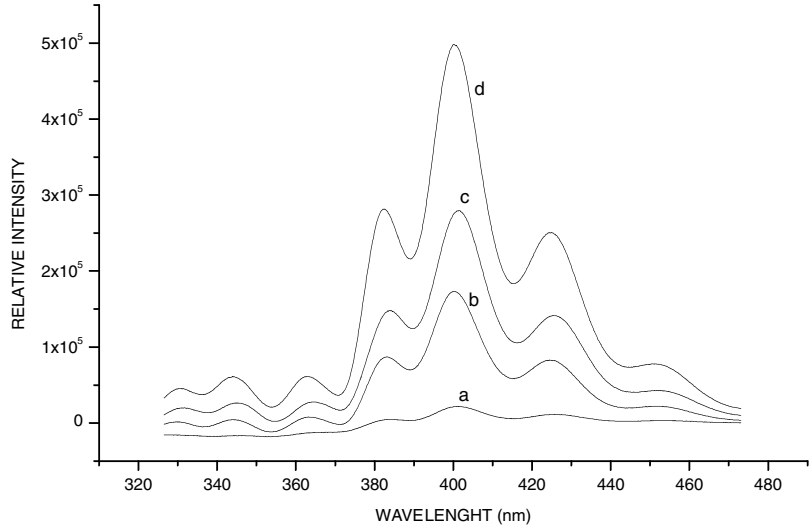


Fig. 6 Variation of the fluoresceine fluorescence spectrum using different optic elements: (a) none, (b) mirror, (c) lens and (d) lens and mirror

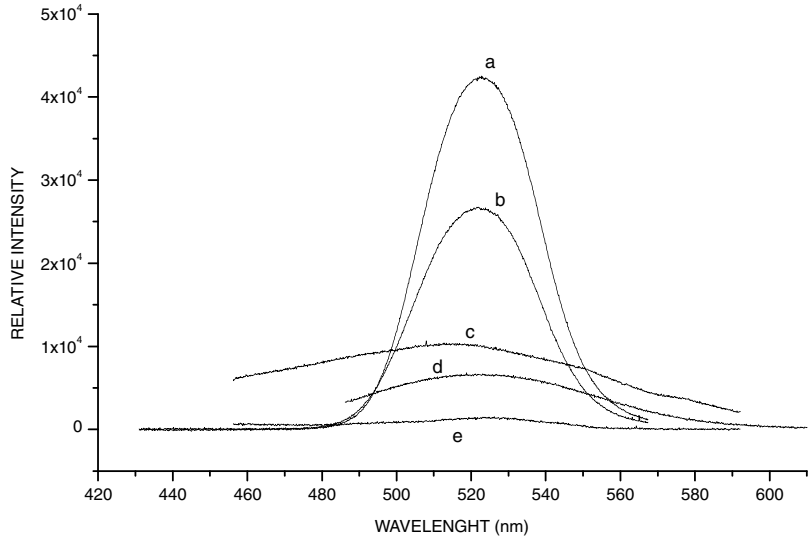


Fig. 7 Variation of the fluoresceine fluorescence spectrum using different sample holders: (a) 1 cm path length, (b) flow cell 4 mm path length, (c) capillary, (d) capillary and 60 x objetive microscope and (e) capillary and 40 × objetive microscope

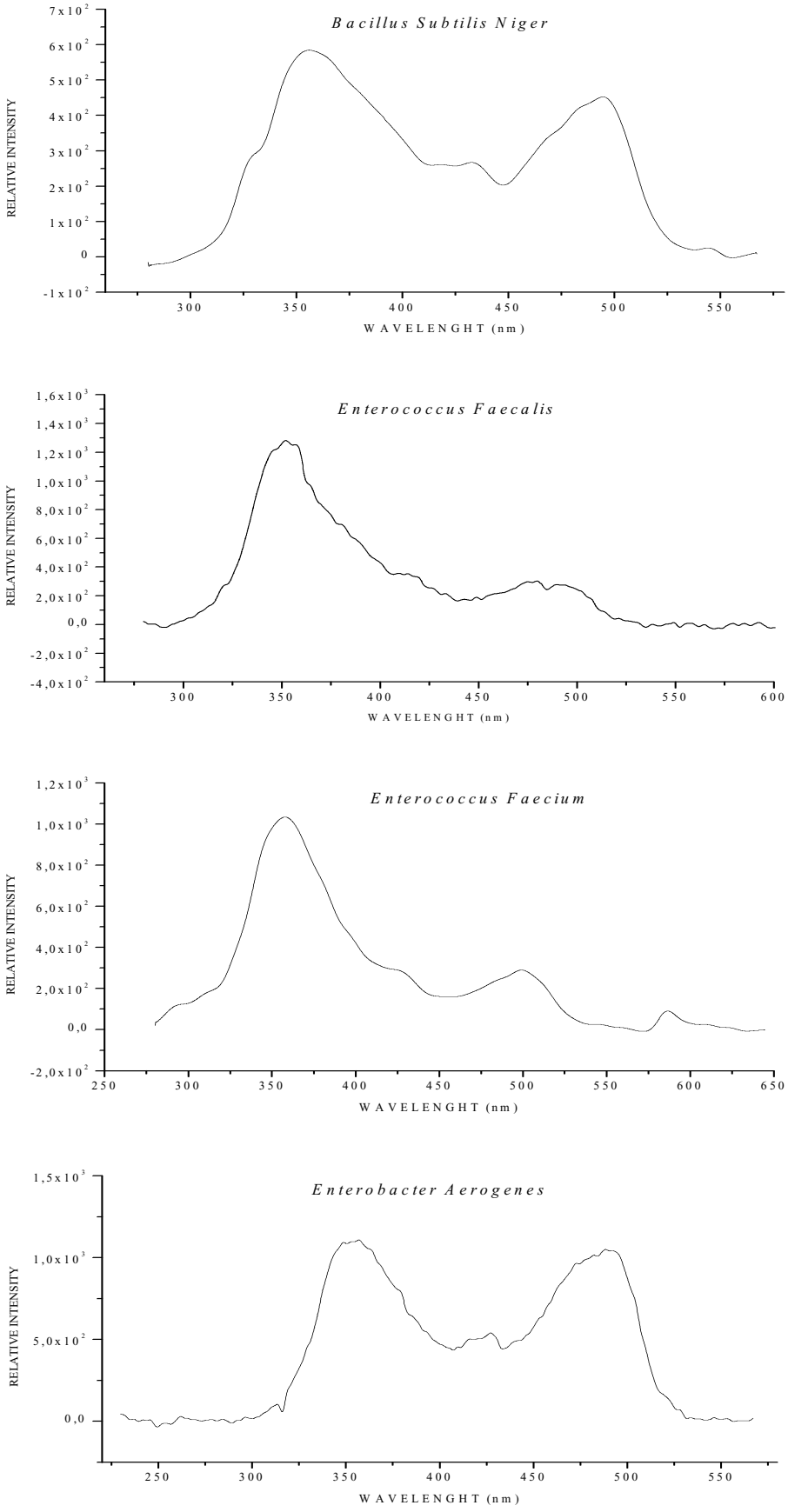


Fig. 8 Bioaerosol fluorescence spectra

Fig. 8 Continued

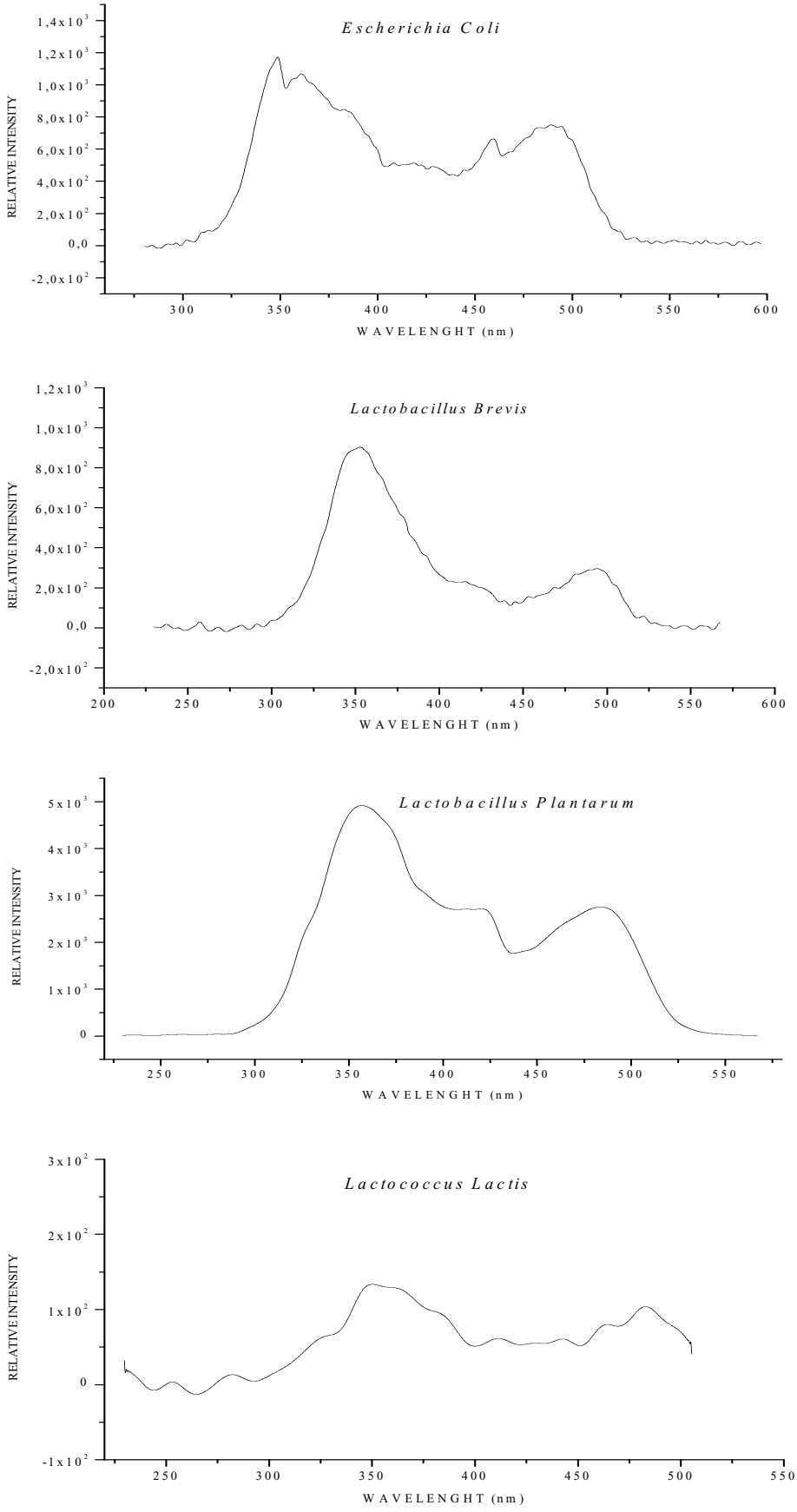
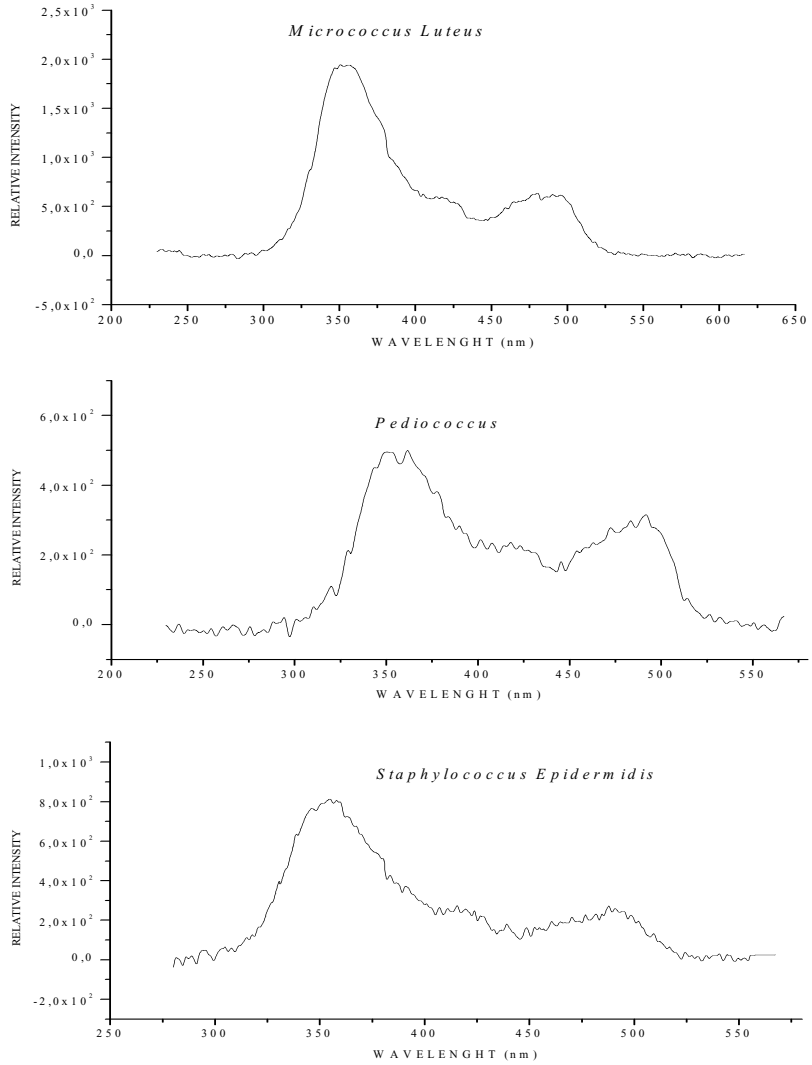


Fig. 8 Continued



Conclusion

The main conclusion is that a laser-induced fluorescence system has been optimised and a small collection of bacteria fluorescence spectra has been presented. All these spectra have been obtained working on the liquid phase. This study is the first of a project based on the use of fluorescence spectra to characterisation of atmospheric bioaerosols.

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References

1. Hinds WC (1999) Aerosol technology: Properties, behavior, and measurement of airborne particles, 2nd edn. John Willey & Sons
2. Parat S, Perdrix A, Baconnier P (1999) Relationships between air-conditioning, airbone microorganisms and health. Bull Acad Nat Med 183(2):327–344
3. Spurny KR (1994) On the chemical detection of bioaerosols. J Aerosol Sci 25(8):1533–1547
4. Ge Z, Wexler AS, Johnston MV (1998) Laser desorption/ionization of single ultrafine multicomponent aerosols. Environ Sci Technol 32:3218–3223
5. Jayne JT, Leard DC, Zhang X, Davidovits P, Smith KA, Kolb CE, Worsnop DR (2000) Development of an aerosol mass spectrometer for size and composition analysis of submicron particles. Aerosol Sci Technol 33(1–2):49–70
6. Cheng YS, Barr EB, Fan BJ, Hargis PJ Jr, Arder DJ, O'Hern TJ, Torczynski JR, Tisone GC, Preppernau BL, Young SA, Radloff RJ (1999) Detection of bioaerosols using multiwavelength UV fluorescence spectroscopy. Aerosol Sci Tech 30:186–201

7. Pan YI, Holler S, Chang RK, Hill SC, Pinnick RG, Niles S, Bottiger JR (1999) Single-shot fluorescence spectra of individual micrometer-sized bioaerosols iluminated by a 351- or a 266-nm ultraviolet laser. Opt Lett 24(2):116–118

8. Seaver M, Eversole JD, Hardgrove JJ, Cary WK Jr, Roselle DC (1999) Size and fluorescence measurements for field detection of biological aerosols. Aerosol Sci Tech 30(2):174–185