APPLICATION OF STANDOFF LIF TO LIVING AND INACTIVATED BACTERIA SAMPLES

Arne Walter[1], Frank Duschek[1], Lea Fellner[1], Karin Grünewald[1], Herbert Tomaso[2], Jürgen Handke[1]

[1] *arne.walter@dlr.de*
Institute of Technical Physics, German Aerospace Center, Langer Grund, 74239 Hardthausen, Germany

[2] *Friedrich-Loeffler-Institut, Institute of Bacterial Infections and Zoonoses, Naumburger Strasse 96a, 07743 Jena, Germany*

Abstract

To minimize the impact of an airborne bio-agent output, sensitive, specific and swift detection and identification are essential. A single method can hardly meet all of these requirements. Point sensors allow highly sensitive and specific identification but are localized and comparatively slow. Most laser-based standoff systems lack selectivity and specificity but provide real-time detection and classification in a wide region with additional information about location and propagation. A combination of both methods allows benefiting from their complementary assets and may be a promising solution to optimize detection and identification of hazardous substances.

Here, we present progress for an outdoor bio-detector based on laser-induced fluorescence (LIF) developed at the DLR Lampoldshausen. After excitation at 280 and 355 nm, bacteria species express unique fluorescence spectra. Upon deactivation, the spectral features change depending on the applied method.

Keywords: Bacteria; Fluorescence, laser induced; Spectroscopy; Standoff detection; Biological sensing and sensors; Hazardous material; Biological agent

1 INTRODUCTION

Terrorist attacks with bio-agents like e.g. the release of the neurotoxin sarin in the Tokyo subway in 1995 [10] and anthrax letters in 2001 [3] or accidental shipment of anthrax contaminated material in 2015 show the importance of effective detection of potentially hazardous substances. Most bio-agents are inexpensive and easy to obtain, grow and deploy. Due to a potential delay between infection and symptoms and the possibility of person-to-person spread, fast detection, localization and identification are crucial for immediate safety and containment measures.

Laser-based detection of hazardous material can be performed from safe distances up to the kilometre range and monitor wide areas in real-time. Operating an outdoor detection system is challenging because environmental material e.g. pollen, dust and diesel in the have to be taken into account. Fog, rain and snow affect laser propagation and stray light while incident sun- and ambient light interfere with the measurement. Furthermore, for use in inhabited areas, the laser radiation has to be eye safe.

1.1 Laser Induced Fluorescence

Established standoff detection techniques detect either plasma emission (Laser induced plasma spectroscopy, LIBS [5, 11]), inelastic scattered light (Raman spectroscopy [11]) or laser induced fluorescence (LIF [6, 11]). LIF spectroscopy yields a higher response signal than Raman spectroscopy and does not need as high excitation energies as LIBS. On the

downside the obtained spectral features are limited. Deployment of multiple excitation wavelengths and time-resolved measurements provide additional information and, combined with sophisticated data analysis, significantly improves discrimination. However, LIF-based identification of bacteria has not yet been achieved. Here, we present and discuss promising data for further differentiation of bacterial species. To date, the combination of fast LIF-based detection, classification and localization and a subsequent directed acquisition and identification of a potentially hazardous sample with point sensors seems to be the most promising solution.

1.2 Bacteria fluorescence

Compared to chemical solutions, spectroscopy on bacteria is more complex. Bacteria differ in size and shapes and occur as single cells or may form clusters, chains or microfilms. They adapt to environmental conditions like nutrient supply and change their metabolic activity to these effects [1]. When exposed to stress, some bacteria, like *Bacillus anthracis*, can reversibly form endospores, dormant structures of high durability and changed constituent composition [8].

Observed bacteria fluorescence is a superposition of several contributing molecules absorbing at the respective excitation wavelengths. For *Escherichia coli (E. coli)*, fifteen potential contributors have been described [7]. Table 1 shows the most contributing fluorophores together with relevant absorption coefficients, mass fractions and estimated fluorescence quantum yields as well as calculated contributions to overall fluorescence at excitation wavelengths of 280 and 355 nm.

Substance	ε(280 nm) l mol^{-1} cm^{-1}	ε(355 nm) l mol^{-1} cm^{-1}	Mass fraction g / g dry weight (%)	Fluorescence		
				Quantum Yield	Calculated contribution at 280 nm (%)*	Calculated contribution at 355 nm (%)*
Tryptophan	5800	0	1.1 (4.0)	0.12	95.9	0.0
Tyrosine	1400	0	2.3 (3.5)	0.003	1.4	0.0
NADH + NADPH	2500	5200	0.048 (0.058)	0.04	0.2	27.1
Riboflavin + FMN	5000	9000	0.011 (0.01)	0.13	0.5	58.6
FAD	30000	9000	0.045 (0.045)	0.013	0.6	11.7
Pyridoxal derivates (unbound)	1000-2200	0-800	0.012 (0.012)	0.01-0.14	0.1	2.6
Ubiquinol	40000	0	0.003 (0.0)	0.018	0.1	0.0
Menaquinol	2700	6000	0.001 (0.003)	0.27	0.0	0.10

Table 1: Overview of the main fluorescing compounds for *E.coli* with spectral characteristics summarized from the work of Hill et al. [7]. Additionally, resulting estimated contribution to the overall fluorescence are displayed. Mass fractions for a mixture of closely related species from the genus *Bacillus* are set in parenthesis for comparison. ε is the decadic molar attenuation coefficient. Note that quantum yields are highly dependent on the local environment of a fluorophore and may vary in magnitudes. *calculated from given values

Since attenuation-coefficient ratios at these wavelengths differ significantly for most specified molecules, different sets of molecules will be probed and may lead to characteristic fluorescence spectra. Promising results of a first advance in bacteria LIF spectroscopy are presented in Ref. [4] and [12].

Upon excitation at 280 nm, *Bacillus thuringiensis* (*B. thuringiensis*) and *E. coli* have shown clearly distinguishable fluorescence spectra [4], as presented in Figure 1. As can be seen from Table 1, below 500 nm, dominant fluorophores should be mainly tryptophan (emission spectrum shown as dashed grey line) and some tyrosine (dash-dotted grey line) whereas the spectra above 500 nm coincide with NADH fluorescence (dotted grey line). The sum of these three fluorophores (solid grey line) was plotted to fit *B. thuringiensis'* fluorescence (solid blue line) with surprising agreement. Excitation at 355 nm produced similar fluorescence spectra which are in good agreement with NADH fluorescence for wavelengths greater than 500 nm. Note that measured bacteria spectra will rarely be just the sum of their components' spectra. Only few molecules within a bacterium contribute to fluorescence, others will reabsorb. Another issue is each molecule's fluorescence quantum yield, which can vary by several magnitudes depending on the local environment. The quantum yields found for molecules in solution can be a hint at best [9].

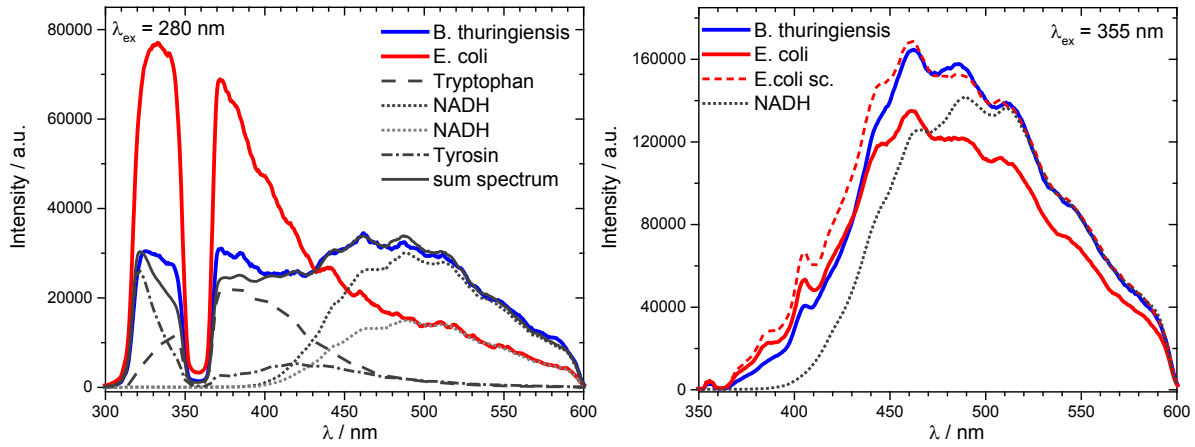

Figure 1: LIF spectra of *B. thuringiensis* (10^7 CFU/ml in PBS) and *E.coli* (10^9 CFU/ml in PBS) upon excitation at 280 nm (left) and 355 nm (right) [4, 12]. E. coli spectrum at 355 nm is scaled for comparison (dashed red line). On the left panel, fluorescence spectra of tryptophan, NADH and tyrosine and the sum of those spectra are plotted (dashed, dotted, dash-dotted and solid grey line), scaled to tentatively fit *B.thuringiensis*. On the right panel, only NADH is plotted (dotted grey line). All spectra are corrected for solvent-contributions.

While *E.coli* and *B. thuringiensis* are distant relatives in the domain of bacteria, the closely related members of the *Bacillus (B.)* genus, *B. subtilis*, *B. atrophaeus* and *B. thuringiensis* can also be distinguished by their characteristic fluorescence [12], as shown in Figure 2.

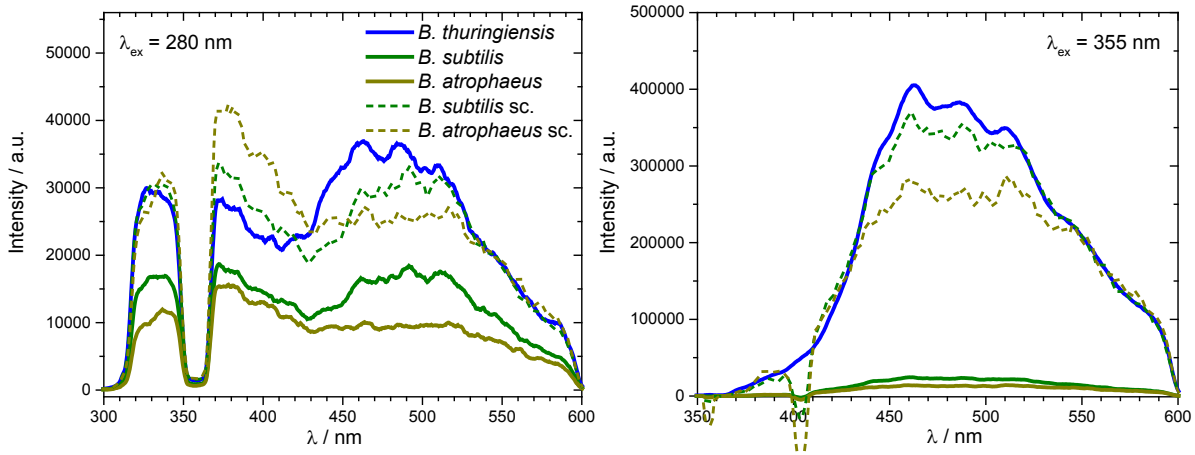

Figure 2: Fluorescence spectra of *B. thuringiensis, B. subtilis* and *B. atrophaeus* (10^8 CFU/ml in PBS) upon excitation at 280 nm (left) and 355 nm (right) [12]. Dashed curves are scaled to match the signal strength of *B. thuringiensis* on the red flank for comparison. Spectra are smoothed and corrected for solvent-interactions.

For proof of principle, LIF standoff detection for classification and identification of bacteria was evaluated using non-hazardous bacterial species as place holder for pathogenic bacteria. Future evaluations will have to be also performed with hazardous species in aerosol chambers at biosafety level 2 and 3 conditions to create a database that will allow differentiation among bacteria genera or even species. In this work, we investigate the influence of deactivation on the fluorescence spectra. Application of inactivated bacteria would facilitate the experimental approach since time consuming and potentially dangerous work with highly pathogenic bacteria could be reduced or completely avoided and data for classification could be gathered conveniently.

2 EXPERIMENTAL SETUP

2.1 Optical setup

To meet the challenges of outdoor detection, the standoff LIF detection system used for the conducted measurements is operated on a free space optical test range at the German Aerospace Center at Lampoldshausen. Chemical, biological and explosive (CBE) substances can be measured at distances from 20 to 135 m under different weather conditions. Laser and detection system are located in an open laboratory whereas the target is positioned outdoors. While experiments can be carried out with both aerosols and liquid phase in a cuvette at a range of distances, all data shown here were collected for suspensions at a distance of 22 m.

Eye safe laser pulses at 280 nm and 355 nm are used to excite the sample. Time-resolved spectra are captured by a gated iCCD camera. The detection system is prepared for background correction and trained pattern recognition software that classifies the sample into coarse classes. The schematic setup of the detection system is shown in Fig. 3.

A Nd:YAG laser supplies pulses of 7 ns pulse length at 355 nm with a repetition rate of 10 Hz. Each second pulse is frequency converted to 280 nm, resulting in an effective repetition rate of 5 Hz with pulse energies of about 10 mJ for each wavelength. Both wavelengths are directed onto the target substance. Fluorescence light is collected by a Newton telescope with an optical diameter of 400 mm. The collected light is split, spectrally filtered to suppress the laser lines and coupled into two optical fibers. One fraction is detected by a photomultiplier tube (PMT) providing information on the wavelength-independent temporal signal of the fluorescence. The other fraction is analyzed by a spectrometer with a resolution of 1 nm spanning a spectral range of 300 – 600 nm and detected by a gated iCCD camera. Time resolved spectra are obtained by a combination of

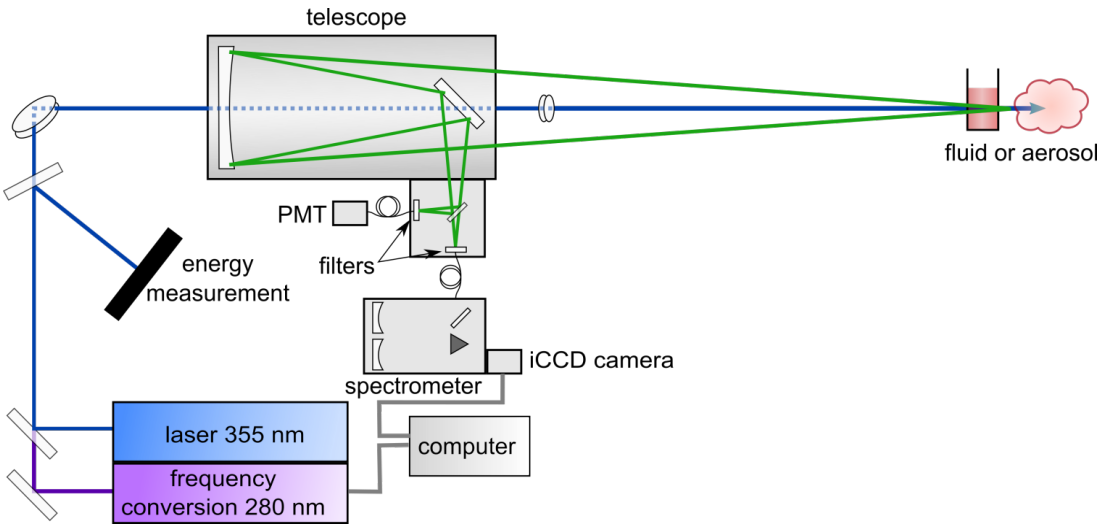

Figure 3: Schematic draft of the optical setup

several consecutive measurements with camera gates delayed relative to the laser pulse. 50 ms after each laser pulse, a background spectrum is recorded to compensate for fluctuations in background radiation and to make measurements more independent from weather conditions. A detailed description of the optical and electronical setup and on data acquisition and processing is given in Ref [6].

2.2 Cultivation and Sample preparation

Bacterial strains: *Bacillus thuringiensis* var. kurstaki strain HD-1 (isolated from insecticide DIPEL), *Escherichia coli* K12[2], *Bacillus atrophaeus* Nakamura 1989 (DSM 7264) and *Bacillus subtilis* (DSM 1970).

Cultivation of the bacteria species was carried out on blood agar plates (nutrient agar 1 obtained by Sifin, Berlin, Germany) supplemented with 7.5 % cattle or sheep blood at 37 °C for 24 h. Colony material was harvested and suspended in phosphate buffered saline (PBS) and stored at 4 °C. The concentration of colony forming units (CFU) per ml was determined by cultivation of 100 µl serially diluted solution in PBS with 0.4 % agar on agar plates at identical conditions. Bacterial suspensions were diluted with PBS to the concentrations measured. Chemical samples (NADH, amino acids) were solved in deionized water, stirred for 1 h and stored at 4 °C over night. Deactivation of bacteria was carried out either by heating at 95 °C for 10 min, autoclaving at 134 °C for 30 min, dilution in Ethanol and gamma radiation at 30 kGy +/- 10 % (done by Synergy Health Radeberg GmbH, Radeberg, Germany).

3 RESULTS

Samples of *B. thuringiensis* [12] and *E.coli* were deactivated by different treatments. The corresponding LIF spectra are presented in Fig. 4. Relative changes of fluorescence intensity calculated from the LIF spectra of Fig. 4 are illustrated in Fig. 5. Exited at 280 nm, fluorescence of *B. thuringiensis* (Fig. 4a) shows minor spectral changes after heating to 95 °C, namely a decrease in fluorescence below and a minor increase above 350 nm (see Fig. 5a). The 355 nm spectrum (Fig. 4b) remains almost unchanged (Fig. 5e). For the autoclaved samples, the spectra show similar changes with a larger increase in overall fluorescence intensity (Figs. 5b,f). Treated with ethanol, the 280 nm spectrum remains unchanged above 400 nm but fluorescence below significantly increases (Fig. 5c). The 355 nm spectrum also expresses an increase for wavelengths below 500 nm (Fig. 5g). Irradiation with gamma rays results in the most drastic changes of the fluorescence spectra. Excitation at 280 nm induces less fluorescence below 350 nm but an increased emission for longer wavelengths (Fig. 5d). Excited at 355 nm, fluorescence is increased for all observed wavelengths, especially below 450 nm (Fig. 5h).

Exited at 280 nm, fluorescence of *E. coli* (Fig. 4c) shows more significant spectral changes than *B. thuringiensis.* After heating to 95 °C, fluorescence decreases below 400 nm and significantly increases above (see Fig. 5a). The 355 nm spectrum (Fig. 4d) shows roughly the same behavior with almost doubled fluorescence intensity between 450 and 500 nm compared to the active sample (Fig. 5e). Autoclaved, the spectra show similar changes as for *B. thuringiensis* but with decreased fluorescence intensity below 400 nm (Figs. 5b,f). After being treated with ethanol, the 280 nm spectrum remains largely unchanged above 450 nm but fluorescence below increases (Fig. 5c). For 355 nm, fluorescence increases for wavelengths below 500 nm (Fig. 5g). Note that upon treatment with ethanol, the spectral changes for *B. thuringiensis* and *E. coli* at 355 nm coincide in shape, while less pronounced for *E.coli*. As for *B. thuringiensis*, irradiation with gamma rays results in the most drastic changes in the fluorescence spectra of *E. coli*. Excitation at 280 nm induces less fluorescence below 350 nm but increased emission for longer wavelengths (Fig. 5d). Excited at 355 nm, fluorescence is increased for all observed wavelengths but especially below 450 nm, just like for *B. thuringiensis* (Fig. 5h).

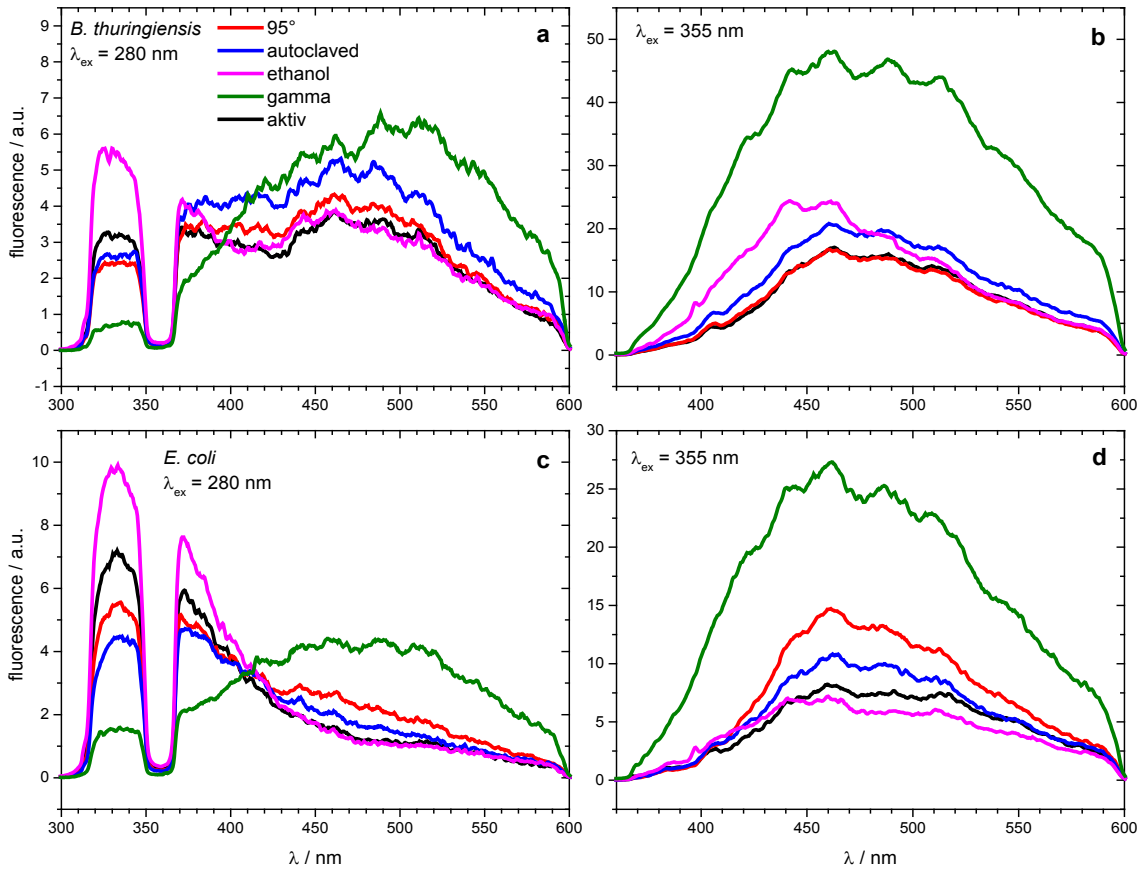

Figure 4: LIF spectra of *B. thuringiensis* (a,b -10^7 CFU/ml in PBS) and *E. coli* (c,d - 10^9 CFU/ml in PBS) excited at different wavelengths after deactivation by different methods

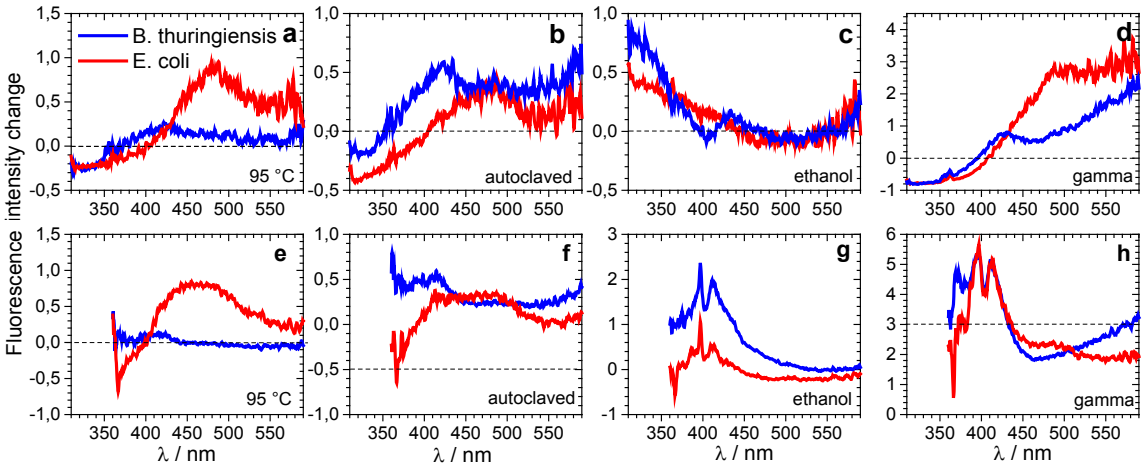

Figure 5: Relative changes in LIF spectra of *B. thuringiensis* and *E. coli* induced by different methods of deactivation excited at 280 nm (a-d) and 355 nm (e-f), calculated from LIF spectra in Fig. 4.

Most methods of deactivation shown here change the fluorescence spectra significantly both in amplitude and in shape. While heating to 95 °C seems quite promising for *B. thuringiensis* since it causes only minor spectral changes at both wavelengths, fluorescence drastically changes for *E. coli.* So far, deactivation does not generally seem feasible to produce optical facsimiles for pathogen bacteria. It may be that certain species respond well to certain treatments. Comparisons of the presented methods indicate a similar spectral respond of different bacteria on some treatments, e.g. deactivation by ethanol (see Figs. 5c,g). If this indication can be confirmed for further species and can be utilized has yet to be tested.

4 CONCLUSION AND OUTLOOK

Hyperspectral LIF standoff spectra of very distant relatives of bacteria as well as of closely related species have been shown for UV excitation wavelengths. The spectral diversity even within three species of a genus was proven to be high enough for safe distinction. Spectral accordance of some fluorophores with parts of the bacteria spectra has been illustrated. It was shown that inactivation methods can have influences on the spectra of *B. thuringiensis* and *E. coli.* While so far no tested method of deactivation can be generally applied, some treatments may work for selected bacteria.

As an outlook, an extension of the classifier for more distinct classification up to identification on the species level is desirable and in perspective, the spectral database [4, 6] will be extended. Investigations in more detailed classification will be carried out. The use of inactivated bacteria for safe handling of hazardous species will be investigated further by applying more deactivation methods on a wide range of species. Furthermore, a more detailed investigation of the spectral contributions of bacteria fluorophores will be performed.

REFERENCES

[1] Abee, T. and Wouters, J.A. (1999). *Microbial stress response in minimal processing.* International Journal of Food Microbiology. 50, 1-2, pp. 65–91.

[2] Clowes, R.C. and Hayes, W. (1968). *Experiments in microbial genetics*. Blackwell Scientific Publications.

[3] Council, N.R. (2011). *Review of the Scientific Approaches Used During the FBItextquotesingles Investigation of the 2001 Anthrax Letters*. The National Academies Press.

[4] Duschek, F. et al. (2015). A fast Hyperspectral Laser Induced Fluorescence application for standoff detection and online classification of biological hazardous materials. *10th Future Security 2015. Security Research Conference. Proceedings* (Berlin, 2015), pp. 97–104.

[5] Duschek, F. et al. (2010). Stand-off detection at the DLR laser test range applying laser-induced breakdown spectroscopy. *Optics and Photonics for Counterterrorism and Crime Fighting VI and Optical Materials in Defence Systems Technology VII*. SPIE-Intl Soc Optical Eng.

[6] Fischbach, T. et al. (2015). Standoff detection and classification procedure for bioorganic compounds by hyperspectral laser-induced fluorescence. *Chemical, Biological, Radiological, Nuclear, and Explosives (CBRNE) Sensing XVI*. SPIE-Intl Soc Optical Eng.

[7] Hill, S.C. et al. (2013). Fluorescence of bioaerosols: mathematical model including primary fluorescing and absorbing molecules in bacteria. *Opt. Express*. The Optical Society.

[8] Koehler, T.M. (2009). Bacillus anthracis physiology and genetics. *Molecular Aspects of Medicine*. 30, 6 (Dec. 2009), pp. 386–396.

[9] Pan, C.-P. and Barkley, M.D. (2004). Conformational Effects on Tryptophan Fluorescence in Cyclic Hexapeptides. *Biophysical Journal*. 86, 6, pp. 3828–3835.

[10] Tu, A.T. (1999). Overview of Sarin Terrorist Attacks in Japan. *ACS Symposium Series*. A.T. Tu and W. Gaffield, eds. American Chemical Society (ACS). pp. 304–317.

[11] Wallin, S. et al. (2009). Laser-based standoff detection of explosives: a critical review. *Anal Bioanal Chem*. 395, 2, pp. 259–274.

[12] Walter, A. et al. (2016). Stand-off detection: distinction of bacteria by hyperspectral laser induced fluorescence. *Proc. SPIE 9824*.