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Faculty of Biomedical Engineering in Kladno
Department of Natural Sciences

DEVELOPMENT OF ACTIVE PART OF OPTICAL FIBER BIOSENSOR USING GENETICALLY MODIFIED ORGANISMS

VÝVOJ AKTIVNÍ ČÁSTI OPTOVLÁKNOVÉHO BIOSENSORU ZA POUŽITÍ GENETICKY UPRAVENÝCH MIKROORGANISMŮ

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Obhajoba disertace se koná dne _____ v _____ hod. před komisí pro obhajobu disertační práce ve studijním oboru Biomedicínská a klinická technika v místnosti č _____ Fakulty biomedicínského inženýrství ČVUT v Praze.

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1. CURRENT STATE OF THE PROBLEM

1.1. Need for a sensor

Chemical sensors are miniaturized analytical devices that can deliver real-time and on-line information on the presence of specific compounds or ions in complex samples. (1) Fiber optic sensors (FOS) are a subset of optical sensors, which use optical fiber in their design. Optical fibers fundamentals and light propagation in optical fibers are described in (2) (3). The advantages of the use of optical fibers in FOS are small dimensions, low attenuation on long distances, resistance to electromagnetic noise, possibility of measuring small volume samples and possibility of performing online measurements in flammable or in normally inaccessible sites. (2)

Biosensors are devices that incorporate a biological sensing element in their design. Biological elements are of more or less complex nature - organisms, tissues, cells, organelles, membranes, enzymes, receptors, antibodies, nucleic acids, and organic molecules could be used as a sensing element. Advantage of biological elements is their biocompatibility compared to an inorganic fluorophore.

Pospíšilová et al. (2015) describe four fiber optic bio-sensors (FOBS) classifications. Enzyme FOBS employ various catalytic enzymes, which are the most widespread type of biosensor. In general, they have great specificity and sensitivity, but the preparation and enzymes purification are time consuming and costly. The glucose sensor by Pasic et al. (2007); hydrogen peroxide, xanthine and hypoxanthine sensor by Spohn et al. (1995); or dopamine sensor by Yuanting et al. (2012) could be mentioned as an example. Whole cells FOBS use microbial cells that react to an analyte. An indicator (pH, oxygen) or optical properties of cells are than the subject of detection (fluorescence, luminescence). Microbial cells are easy to manipulate, more viable and stable in-vitro. The toluene biosensor by Kalabova et al. (2013) could be mentioned as an example. Immunoassay FOBS exploit the binding between an antibody and antigen, which is detected through a fluorescent label or by evaluation of reflectivity or refractive index. The fiber optic SPR probe by Mai et al. (2019) could be mentioned as an example. Nucleic acid FOBS use the single-stranded DNA that forms double strands with a complementary strain, usually labeled with an optical indicator. The adenosine sensor by Xiyu et al. (2019) could be mentioned as an example. (2) (4) (5) (6) (7) (8) (9)

“According to their mode of action, optical biosensors have been subdivided into five subgroups: (a) plain fluorometric sensors; (b) direct and indirect indicator-mediated chemical sensors; (c) direct enzymatic biosensors; (d) indicator-mediated enzymatic biosensors; and (e) affinity biosensors.“ Typical optical measurement methods include absorbance or reflectance, fluorescence, bioluminescence or chemiluminescence. (2)

Wang and Wolfbeis (2020) published an extensive updated review of Fiber-Optic Chemical Sensors and Biosensors. (10)

Whole cell biosensors allow us to obtain functional information (effect of stimulus on a living system, effect of a substance on other aspect of cellular metabolism, cell toxicity, etc) and qualitative or quantitative analytical information (what substance is present and in what concentration). Sensitivity of a cell to a substance is determined by its receptor/ligand binding constant. A relatively new approach is to genetically modify a cell strain to increase its specificity and sensitivity to a specific substance. Monitoring the state of cells to obtain desired information is carried out either on its energy metabolism - measurement of pH, O_2 consumption, CO_2 production, lactate production or it uses specific features of some cells such as potential on nerve cells or bioluminescence. Whole-cell biosensors employing GMO is reviewed by Wei et al. (2015). (11)

Among immobilization techniques, cell adsorption and **entrapment** into silica gel by sol-gel method possess the properties of transparency, chemical inertness and biocompatibility which are of primary importance for optical sensors. This method was used to entrap *P. putida* TVA8 in a drop of silica gel on tapered element to form a biorecognition layer of a biosensor that detected toluene in water repeatedly for over two weeks. (12) The **adsorption** of cells onto solid surfaces is one of the initial steps of microbial biofilm formation, which can be regarded as a natural method of whole cell immobilization. (13) In order to predict and understand the process of microbial adsorption onto solid surfaces, different physicochemical models can be applied. (13) (14) The key biofilm characteristics were described by Donlan (2002) or Kokare et al. (2009). (15) (16)

The use of tapered **optical fiber element** (OFE) (see Fig. 1) in a biosensor is a novel approach which increases the photon detection efficiency and amplifies the weak bioluminescent signal, produced by bioreporter organisms, by increasing the number of cells (light sources) on the OFE cross-section. The cross-section binds light into the fiber most

effectively in comparison to the side of the OFE. The narrow end of the OFE is than connected to a photon-counter detector.

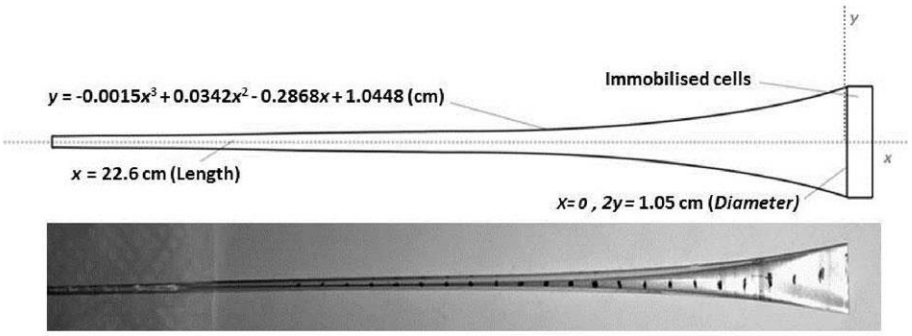

Fig. 1: Tapered optical fiber element "0" with marks for measurement of diameters. (17)

The design of whole cell biosensor, encompassing OFE, was chosen for its low cost in comparison to lengthy chemical analysis, simplicity and relatively fast analysis. It allows us to preform continuous monitoring in remote sites, it is possible to make the biosensor small, compact and it could detect several measurands at the same time (18). Furthermore, it could measure the total bioavailability of a pollutant rather than its free form. Nevertheless, the chosen approach also has potential drawbacks, such as worse handling, limited lifetime of living components, lower sensitivity in comparison to chemical analysis, response time to an analyte from tens of minutes up to hours, and legal obstacles to employ GMO in the environment or living organism, but there already have been several exceptions (19) (20).

The principle of sensing and detection could be used in general, using different microorganisms in different fields and applications. This opens endless possibilities for fast and inexpensive determination of numerous analytes in medicine.

1.2. Analytes of interest and their detection

Biomedical Engineering is a wide interdisciplinary scientific discipline. One of the areas of biomedical engineering interest is environmental pollution, its remediation and the effect of pollutants on human health. Chemicals with adverse health effects, recognized by multiple authorities (e.g. World Health Organization or United Nations Environment Programme), are selected due to their toxicity in low concentrations, bioaccumulativity, persistency, or carcinogenicity (21) (22). Some of the most frequently found pollutants, which pose a serious

environmental concern, are benzene, toluene, ethylbenzene and the xylenes (BTEX), widely found in landfill leachates or petroleum products leakage sites. (23)

Conventional chromatographic and mass spectrometry analyses of pollutants in aquatic environments are costly and time consuming. Whole-cell biosensors, which produce light selectively in the presence of a specific analyte, represent a low-cost and fast in-situ alternative for established BTEX detection. Biological sensing elements are whole cells which have been constructed to produce light signal in response to a compound of interest in their environment. Such bioreporters have been engineered since 1992 (24), and the proof-of-principle of detection has been demonstrated numerous times (25) (26) (27). The past decade experienced a growth of whole cell bioreporters applications in laboratory and under controlled conditions (28) (29) (30). The first genetically engineered microorganism allowed to be released in the field to monitor bioremediation potential was *Pseudomonas fluorescens* HK44 in 2001 (19). Recently, smartphones (31) and drones (32) have been used as whole cell biosensor devices encompassing bioluminescent bioreporters. Nevertheless, the main reason for limited use of engineered bioreporters is legislative regulation against the environmental release of genetically manipulated organisms (20).

Continuous monitoring with whole cell biosensors requires repeated inoculation or immobilization of cells on the sensing element (19) (33). Formation of a biofilm layer tightly attached to an OFE surface, without an immobilization matrix, does not involve any dropping or printing machines, and minimize loss of the detected bioluminescence signal in comparison to entrapment of cells in polymers, silica gel or plasma deposited films (34) (7). The adhesion of living cells to solid surfaces is influenced by the surface properties of both interacting entities, their motility and properties of the surrounding environment. Targeted modification of the surface properties of solid materials can significantly enhance the adsorption of cells (35).

Bioreporter cells immobilized on the tip of an optical fiber might serve for continuous long-term measurements in small volume samples and remote localities and, unlike electrical sensors, might be applied in places with an explosive atmosphere. Small dimensions of an optical fiber allow to immobilize only a small number of living cells, which provide low bioluminescence intensity, resulting in low biosensor sensitivity. Optical fiber element was developed to increase the signal intensity by increasing the number of light sources (cells) on its wider end (Ø 1 mm–1 cm), where the light coupling efficiency is maximized. (36) (2)

This work demonstrates the adhesion of *P. putida* TVA8, microorganism producing bioluminescence upon metabolizing aromatic hydrocarbons, to an OFE surface after chemical treatment. This active part of biosensor was immersed in toluene solution, positively induced bioluminescence was than measured daily in a light-tight box. Repetitive long-term use of the whole-cell sensor with the biorecognition layer was tested in over 135 days trial. Bioluminescence of the biorecognition layer was also induced in a real polluted water sample. Repeatability of preparation of the biorecognition layer was tested in a trial with five OFEs with different geometries, and an ideal shape of an OFE was calculated. Author also immobilized constitutively bioluminescent bioreporter *E. coli* 652T7 on OFE treated with polyethyleneimine and induced the microorganism in Laurea-Bertani medium (LB) medium.

1.3. BTEX group

Benzene, toluene, ethylbenzene and xylenes (Fig. 2) are organic aromatic hydrocarbon chemicals. They belong to volatile organic compounds that have high vapor pressure at room temperature. BTEX are associated with petroleum and its products. They can be present in gasoline, rubbers, plastics, paints or even in car exhausts or in cigarette smoke. Besides these human made products, these compounds can also be found also in petroleum production and use sites as environmental contaminants. Natural sources of BTEX are gas and petroleum deposits, emissions from volcanos or forest fires. (37)

BTEX are partially water-soluble, thus it is obligatory to monitor BTEX concentrations in the drinking water, industrial areas and other risk localities (benzene – 1.84 g/L at 30°; toluene - 0.52 g/L at 20 °C; ethylbenzene - 0.15 g/L at 20 °C; xylenes – 0.12 g/L at 20°).

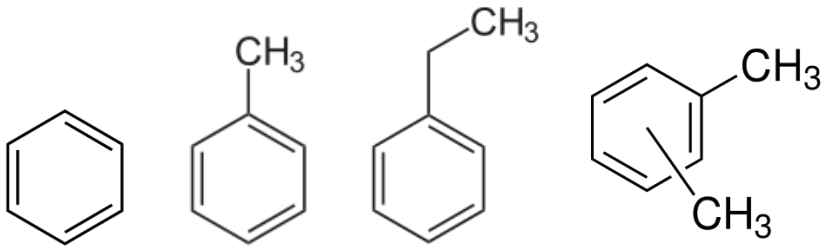

Fig. 2: BTEX, molecules of benzene, toluene, ethylbenzene and xylene respectively from the left

The following effects of BTEX on human health can be distinguished: Short term effects include skin and sensory irritation, central nervous system problems (tiredness, dizziness, headache, loss of coordination) and effects on the respiratory system (eye and nose irritation). Long term effects include kidney, liver and blood system problems. Chronic exposure to benzene compound can lead to leukemia and cancers of the blood-forming organs. (38)

Concentrations of BTEX compounds in the environment range from 0.1 to over 100 ppb in air. Concentration in ground water is generally below 3 ppb, but in polluted sites it can reach values over 3500 ppb. Higher concentrations are generally detected in urban areas, high traffic density areas, gas stations, and daily consumer products (especially cigarette smoke). Table 1 shows typical reported concentrations of BTEX in water. (37)

Table 1: Reported BTEX concentrations in water (37)

[ppb or µg/L]	**Benzene**	**Toluene**	**Ethylbenzene**	**Xylenes**
Surface water	<0.1 – 2.1	<1 - 15	<0.1 - 1.8	<0.1 - 1.2
Contaminated surface water	Up to 100	NA	Up to 15	Up to 32
Groundwater	<0.1 - 1.8	<1 - 100	<0.1 - 1.1	<0.1 - 0.5
Contaminated groundwater	Up to 330	Up to 3500	Up to 2000	Up to 1340
Drinking water	<0.1 - 5	<1 - 27	<1 - 10	<1.0 - 12

Public health guidelines, based on an acceptable daily intake, were published by numerous agencies operating worldwide – i.g. World Health Organization Drinking Water Guidelines (WHO DWG); Queensland Public Health Regulation (QHPR); Australian Drinking Water Guidelines (ADWG); United States National Primary Drinking Water Standards (US NPDWS). Some of the guideline values are listed in the Table 2. (37)

Table 2: Water BTEX guidelines (37)

[ppb or µg/L]	**WHO DWG**	**QHPR**	**ADWG**	**US NPDWS**
Benzene	10	1	1	5
Toluene	700	800	800	1000
Ethylbenzene	300	300	300	700
Xylene	500	600	600	10000 (total xylenes)

Regulation (EC) No 1907/2006 of the European Parliament and of the Council sets toluene concentration limits for work environments at 680 µg/L in freshwater environment. The regulation also defines the acute toxicity to algae and daphnia (6-245 mg/L within 1-2 days) and fish (7.63 mg/L within 4 days); and chronic toxicity to fish (5.44 mg/L within 7 days). Taiwanese agencies have set acceptable limits for toluene in drinking water and groundwater at 7.6–10.9 µM (900 µg/L). (39)

2. GOALS OF THE DISRTATION

The author proposes a new design of active part of optical fiber biosensor involving OFE and GMO. The following steps were listed to achieve a functional biosensor of toluene:

1. To suggest an approach for developer of active part of optical fiber biosensor based on literature research.
2. To construct new active layer of the biosensor in form of biofilm and test different available bioreporter cells in the setup.
3. To characterize the designed active part of optical fiber biosensor using of laboratory and microbiological procedures and test it on a real polluted water sample.
4. To discuss results for the six available optical fiber elements and suggest future research goals.

Motivation for the elaboration of this dissertation thesis was the need for a cost-effective detection system for online monitoring of environmental pollutants in remote localities, which affect human health.

Previous research at the Faculty of Biomedical Engineering in Kladno, focusing on environmental pollution, was devoted to immobilization of bioreporter organisms on optical fibers using organic or inorganic matrices and on amplifying week bioluminescent signal produced by the immobilized bioreporters, guided by an optical fiber to a detector (H. Kalabova, G. Kuncová, T. Ishizaki). (7) (12) Author of this thesis pushed this research further by determining the ideal properties of the used optical fiber element and by immobilizing bioreporter organisms in the form of biofilm without use of an immobilization matrix, which further increased

the detected bioluminescent signal. Author also tested several different bioluminescent bioreporters in the biosensor design. There are numerous variables that affect the overall response of the developed sensor. Elaboration of every imaginable property would result in several separate dissertations. The goals listed above are fundamental for this research and dissertation.

Two subjects, academic and commercial, were interested in the researched biosensor principle and design, namely the Research Associate Professor of University of Tennessee in Knoxville (UTK), whose research areas are cellular reporter systems for biomedical imaging with ancillary applications in environmental monitoring; and a Czech water treating company (requested to be unnamed) who provided a real polluted water sample for testing the developed biosensor.

3. METHODS

This chapter describes in detail the individual performed experiments, methods and procedures in the same order as they were performed during the experimental work. All experiments were performed at two workplaces authorized for manipulation with genetically modified organisms, namely the laboratory of the Institute of Chemical Process Fundamentals of the Czech Academy of Sciences in Praha-Suchdol (ICP) and the laboratory of partner Center for Environmental Biotechnology of the University of Tennessee in Knoxville, USA (UTK).

3.1. Used microorganisms

Pseudomonas putida **TVA8** is a genetically modified strain, tailored to produce bioluminescence at about 500 nm in the presence of aromatic hydrocarbons (e.g. BTEX contaminants). It was constructed by introducing the *tod-luxCDABE* fusion directly into the chromosome of *P. putida* F1 strain by a constructed transposon. *P. putida* TVA8 was first constructed at the UTK. (40)

Saccharomyces cerevisiae **BLYR** are unicellular and saprotrophic fungi. They divide asymmetrically by process known as budding. Their colonies grow rapidly and mature within

3 days. BLYR strain, used in this study, was created by inserting pUTK401 and pUTK404 plasmids into *S. cerevisiae*, which already contained human AR (hAR) gene in its chromosome. Plasmids encode luciferase enzyme together with necessary aldehyde substrate for its function. *S. cerevisiae* BLYR is constitutively bioluminescent reporter for monitoring of toxicity of chemicals (bioluminescence at about 500 nm). (41)

***Escherichia coli* 652T7** is characterized as a gram negative, facultatively anaerobic, coliform bacteria with a shape of rod and size of about 2 µm. It is considered as one of the most diverse bacterial species. *E. coli's* 652T7 genome contains a plasmid-based phage lambda T7 promoter fusion to the *P. luminescens luxCDABE* gene cassette. Genes encode luciferase enzyme together with necessary aldehyde substrate for its function. This bioreporter bacterium produces bioluminescence constitutively at 500 nm. Toxicity is expressed as inhibition of bioluminescence relative to control samples. (42)

For the purpose of this work all the microorganism strains were kindly donated by the center of Environmental Biotechnology, UTK.

3.2. Determining surface properties of quartz glass and microorganisms

With the aim of proposing a surface modification of the quartz glass (Suprasil) to ameliorate cell adsorption, the zeta potentials and contact angles of both the cells and the quartz glass were measured. The surface properties of the *P. putida* TVA8 cells, in the form of algal layers on membranes filters, and the APTES-modified quartz were characterized by contact angles (CA). Bacterial cells were deposited on a filter (nitrate cellulose membrane, 0.45 µm pore size, 47 mm diameter, Whatman Sigma–Aldrich, USA) under negative pressure. The microbial lawns thus obtained were then deposited on agar plates to stabilize the moisture content, then were fixed on a microscopic glass slide and allowed to dry for 30 min. The CA measurements of both the algal lawns and glass slides were performed by the sessile drop technique (volume of ≈ 3 µL) using a CAM 200 goniometer (KSV Instruments, Finland). The measurements were performed at 25 °C with three test liquids (water, formamide, 1-bromnaphtalene), readings were taken after 0.5 s of deposition, and each sample was tested ten times. The total surface tension and its components, and the values of the free energy

of interaction between cells and carrier in water were calculated in accordance with van Oss et al. (1995). (43)

The zeta potential (ZP) of the bacteria was measured using a Zetasizer Nano-ZS (Malvern, UK) in LB medium at pH 7. The surface charge of APTES-modified quartz was determined in an adjustable gap cell on SurPASS (Anton Paar, Austria) in contact with 170 mM KCl (ionic strength of LB medium) at pH 7. For the zeta potential determination, a streaming current approach and the Helmholtz–Smoluchowski equation were used. (43)

3.3. Chemical modification of optical fiber element

(3-Aminopropyl)triethoxysilane (APTES) - Surface modification had the purpose of increasing surface charge of the OFE, so the cells would more likely adhere to its surface and create a biofilm-like layer. OFE was first washed with detergent and rinsed with deionized water. OFE was than immersed in a piranha solution at 70 °C for 30 min, washed in deionized water and dried at 110 °C for about 1 h. The OFE was than immersed in a mixed solution of (3-Aminopropyl)triethoxysilane (APTES) (5 mass %) in dry toluene at ambient temperature for about 24 h. Lastly the element was rinsed with toluene and acetone and dried at 110 °C for about 1 h.

Polyethylenimine (PEI) – This was an alternative modification technique which was tested when the APTES modification was not sufficient. OFE was first washed with detergent and rinsed with deionized water. OFE was than immersed in a piranha solution at 70 °C for 30 min, washed in deionized water and dried at 110 °C for about 1 h. After drying, the OFE was immersed in 0.2% solution of PEI in deionized water for 30 min, and then air-dried.

3.4. Immobilization of microorganisms

***P. putida* TVA8 -** The modified end of the OFE was fixed vertically in a flask filled with 150 mL of LB containing kanamycin (10 gL^{-1}) and overnight culture of *P. putida* TVA8 (1 mL). The cells were left to grow and adsorb on the element end in the shaker for 4 days, at 25 rpm and at 28 °C. Previous experiments proved this is the minimal time needed for *P. putida* TVA8 cells to be adsorbed. (17)

***E. coli* 625T7 -** Growth of *E. coli* 652T7 on the APTES modified OFE surface was inadequate. Addition of $FeCl_3$ to the growth medium with OFE in the final concentration of 150 µM, which was reported to ameliorate adhesion of cells by decreasing the separation distance (44), was unsuccessfully tested. The PEI modified surface was attempted as an alternative. A 20 mL aliquot of an overnight culture of *E. coli* 652T7 in 150 ml LB_{kan} medium was centrifuged at 3000 g for 5 min. The pellet was resuspended in 20 mL MSM and centrifuged again at 3000 g for 5 min. The pellet was then resuspended in 20 mL of 0.2% PEI in MSM and left in a shaker for 30 min at 100 rpm and 28 °C. Finally, the culture was centrifuged at 3000 g for 5 min and the pellet resuspended in 20 mL of MSM. (45) The wider end of the PEI modified OFE was then immersed in the 20 mL suspension of *E. coli* 652T7 and shaken at 50 rpm for 30 min and 28 °C.

***S. cerevisiae* BLYR -** Growth of *S. cerevisiae* BLYR on the APTES modified OFE surface was inadequate. Addition of $FeCl_3$ to the growth medium with OFE in the final concentration of 150 µM was unsuccessfully tested. The PEI modified surface was attempted as an alternative. A 20 mL aliquot of an overnight culture of *S. cerevisiae* BLYR in 50 ml YMM medium was centrifuged at 3000 g for 5 min. The pellet was resuspended in 20 mL MSM and centrifuged again at 3000 g for 5 min. The pellet was then resuspended in 20 mL of 0.2% PEI in MSM and left in a shaker for 30 min at 100 rpm and 28 °C. Finally, the culture was centrifuged at 3000 g for 5 min and the pellet resuspended in 20 mL of MSM. The wider end of PEI modified OFE was then immersed in the 20 mL suspension of *S. cerevisiae* BLYR and shaken at 50 rpm for 30 min and 28 °C.

3.5. Measurements of induced bioluminescence

The thinner end of the OFE was connected to a light guiding tube or directly to a photon counter placed in a light-tight box, which minimized noise background in the measured signal (Fig. 3). The wider end with adsorbed cells was fixed 4 ± 1 mm from the bottom of a 50 mL glass beaker, filled with 10 mL of induction solution. Reflective aluminum foil was placed underneath the beaker. The wide face of the OFE was pointing down to prevent a sediment formation. Every day, the end of the OFE was gently washed with MSM using a pipette and re-immersed in a fresh induction solution. The exceptions were the 1–4 day pauses for weekends and holidays, when the element was immersed in the induction solution for more than 24 h.

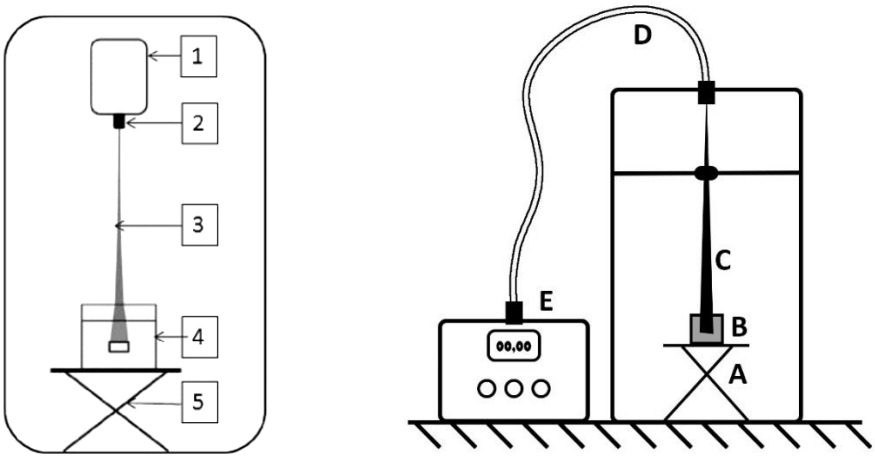

Fig. 3: Experimental set-ups for monitoring bioluminescence, used at ICP (left) (21) and at UTK (right), 1,E-photon counter; 2-SMA connector; D-light guiding cable; 3,C-OFE; 4,B-induction solution; A,5-stand

Intensity of the bioluminescence measured with Perkin-Elmer photon counter at ICP was recorded continuously for about 18 h (10 s integration times). Temperature in the measurement box varied between 25 °C and 29 °C. Due to high noise, the raw data were smoothed in OriginPro software.

Bioluminescence measured with Oriel 7070 measurement system at UTK was recorded with a web camera and values were registered manually every 20-60 min. Accelerating voltage of the photon multiplier tube was set to 850 V and the generated current was then read from the Oriel 7070 detection system in nano-Amperes (nA). Standard laboratory temperature was measured to be 21 °C.

3.6. Scanning electron microscope visualization

SEM was used to verify the attachment of *P. putida* TVA8 to APTES modified OFE. Since the OFEs could not be processed for SEM imaging, quartz cones were used as an alternative. Quartz cones were modified with APTES and *P. putida* TVA8 cells were immobilized as explained above. Surface modified quartz cones with adhered cells were placed in a 50 mL beaker with 30 mL of toluene induction solution. Bioluminescent signaling by the cells was verified by taking light measurements in a Perkin-Elmer IVIS Lumina K imaging system. After two days of immersion, SEM imaging was performed. The quartz cones with immobilized cells were fixed in McDowell–Trump Fixative (Fischer Scientific), gold coated and then viewed in a Zeiss Auriga SEM. Samples of biofilm grown for 2 days and 130 days were compared.

4. RESULTS

4.1. Tapered Optical Fiber Element

The OFE was designed to amplify a week bioluminescent signal by increasing the number of light sources (bioreporter cells) on its face. The shape of OFE contributes to the amount of bioluminescence transmitted from the bioreporters to a detector. Mathematical model was developed by Kalabova et al. (2018) with the aim to optimize OFE parameters and maximize its light transmittance (T), which represents the fraction of light transmitted through an OFE to a detector. The amount of transmitted light is represented by the OFE efficiency, which can be calculated as a product of transmittance and number of 1µm cells which could be fit on wider end of an OFE. Because this concept of OFE efficiency is dependent on the size of a certain microorganism strain, author of this thesis proposed a more generalized model. Author used OFE face area, instead of the cell count, to calculate planar OFE efficiency (equation *A)*. This concept allows to compare OFE efficiencies without considering different immobilized microorganisms or even other biological sensing elements in general (i.e. bacteria and yeast; or bacteria and enzymes). Note that this concept doesn't account bioluminescence intensity produced by a microorganism strain. (36)

$$E = T * A \qquad (A)$$

Where E is OFE efficiency, T is OFE transmittance and A is area of wider end of an OFE.

The model suggested that the best place to immobilize light sources is on the very surface of the OFE face. Bioreporters immobilized in a matrix are separated by a small distance from the OFE (Kalabova et al. (2018) immobilized bioreporters in 2-3 mm thick silica gel), thus it is advantageous to immobilize the bioreporters in a form of biofilm-like layer on the glass surface. Bioreporters immobilized on the OFE side wall does not significantly contribute to the detected signal (<1 %) due to the laws of geometrical optics. (36) (2)

Using the previously developed software (36), T was calculated for several theoretical OFE geometries with the same length, Dmin, and Dmax (Fig. 4, left). Increasing T (Fig. 4, right) of OFE shapes from the most curved to nearly linear confirmed the assumption that the best shape of an OFE is a frustum cone. The limits to this construction are the numerical aperture of an OFE (refractive index of OFE material and refractive index of cladding material or air),

aperture of a sensor or diameter of an optical fiber to which is the OFE connected (maximal output diameter), and maximum possible length that would maximize the size of the OFE face and thus the number of light sources. Kalabova et al. (2018) compared two different OFE geometries and conditions under which they were produced. Nevertheless, the provided information doesn't suggest the ideal drawing parameters, thus further experiments are needed (36).

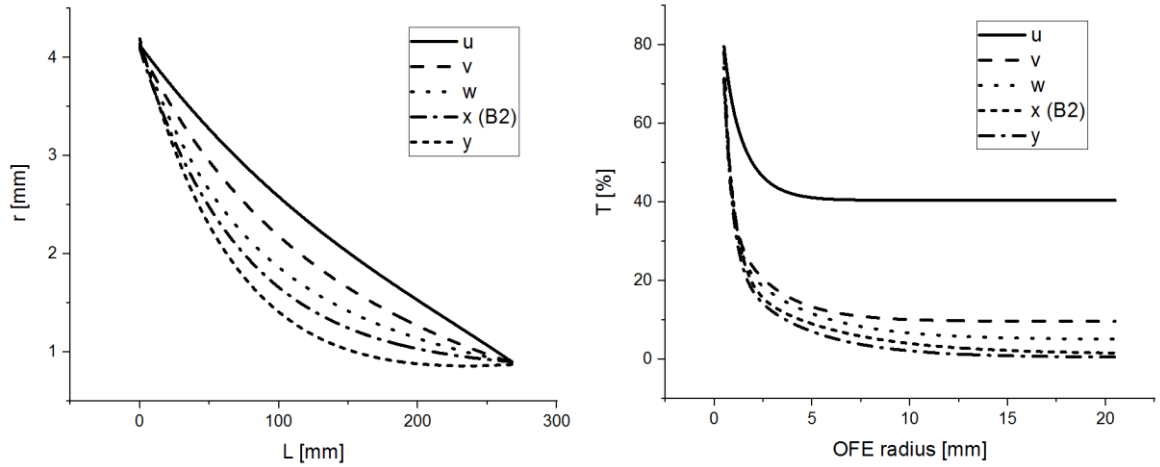

Fig. 4: Geometrical shape of theoretical OFEs (u, v, w, x, y) with identical Dmin and Dmax, but different bends (left), OFE "x" is identical to OFE "B2" used in the experiments (left); calculated Transmittance vs. OFE radius for each theoretical OFE (right).

Table 3 compares calculated transmittance, cell number and OFE efficiency of five available OFEs. The theoretical number of cells on the face of each OFE was determined based on the assumption that the cells are spheres with 1 µm diameter, organized in one layer on the surface of an OFE face (36). OFE efficiency was calculated as a product of the cell number and the OFE transmittance (2).

Table 3: Calculated transmittance, cell number and OFE efficiency

OFE	Transmittance [%]	OFE face area [mm²]	Number of cells	OFE efficiency *10⁵	OFE efficiency planar [mm²]
A	1.41	19.4	2.47 x 10⁷	3.48	0.27354
B1	1.62	13.2	1.68 x 10⁷	2.72	0.21384
B2	2.21	13.2	1.68 x 10⁷	3.71	0.29172
C	1.51	7.26	0.92 x 10⁷	1.39	0.10963
D	5.00	18.47	2.35 x 10⁷	11.75	0.92350

4.2. OFE surface - contact angles and zeta potential

Table 4: Zeta potential of tested samples

Material	Zeta Potential [mV]
Quartz	-21±2
APTES quartz	-3±0.4
P. putida TVA8	-15.6±0.6

Table 5: Contact angles of tested samples for 3 different liquids

Material	Water [°]	Formamide [°]	1-Bromonaphtalene [°]
Quartz	24.6±1.1	19.1±0.8	33.8±2.2
APTES quartz	71.8±2.5	63.2±1.7	30.3±1.6
P. putida TVA8	28.9±0.6	41.2±0.8	45.5±1.4

In the thermodynamic approach to microbial adhesion, the adhesion between solid surfaces in water are energetically favorable when $\Delta G_{TOT} < 0$ and unfavorable when $\Delta G_{TOT} > 0$. The thermodynamic approach does not include the role of long-range electrostatic interactions, hence it is only valid at close contact (Bos et al., 1999). An unfavorable (positive) total adhesion energy balance (Table 4-5) was obtained for both bacteria–water–quartz (33.3 mJ m^{-2}) and bacteria–water–APTES quartz (13.5 mJ m^{-2}) systems. This conflicts with the P. putida TVA8 cell adsorption observed onto APTES quartz. (14)

Since the thermodynamic model was not able to predict the adhesion of the bioreporter cells onto the APTES quartz, the extended DLVO (XDLVO) theory was used subsequently. This combines the conventional non-covalent Liftshitz–van der Waals (LW) and electrostatic (EL) interactions with the Lewis acid–base (AB) interactions (van Oss, 2003). Given the rod-shaped cells, the simulations in accordance with the XDLVO theory were made for the cylinder-flat plate interactions (Adamczyk, 2006), the Hamaker constant (van Oss, 2006) was estimated from the ΔGLW value (Table 4-5) and the characteristic decay length of 0.6 nm for AB interactions in water was used (Bos et al., 1999). The profile of total interaction free energy (G_{TOT}) vs. the separation distance predicted favorable energy balances for adhesion of the *P. putida* TVA8 bioreporter cells to the APTES quartz with a total absence of potential energy barriers (Fig. 5). This XDLVO model prediction supports the experimental observations of rapid bioreporter cell adsorption onto APTES quartz. At the same time, the XDLVO model prediction for *P. putida* TVA8 adhesion to unmodified quartz is characterized by the presence of a high energy barrier

(6911 kT at 0.5 nm; Fig. 5). In the frame of the colloidal interaction model (XDLVO), this energetically unfavorable barrier prevents close contact between the bacteria and the quartz surface. This model prediction was confirmed by the absence of *P. putida* TVA8 adhesion to unmodified quartz. (14) (46) (47) (14)

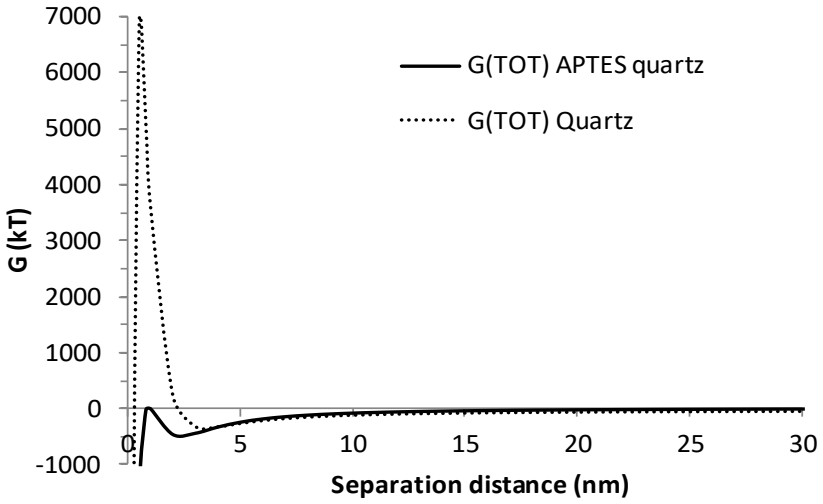

Fig. 5: Total free energy of interaction (G_{TOT}) as function of separation distance between bacteria P. putida TVA8 and surface of either quartz or APTES modified quartz as calculated according to XDLVO theory.

It was experimentally proven, that the *P. putida* TVA8 does not spontaneously adhere onto unmodified quartz surface. The measured Zeta potential of the unmodified glass and reporter cells has a negative surface charge. To support the cell adhesion to the bare OFE, the quartz surface was modified by APTES, which added aminopropyl functional groups to its surface. This increased the original charge from –21 mV to –3 mV. *P. putida* TVA8 cells were than more-likely to get closer to the quartz surface and colonize it. The modification also led to increased surface hydrophobicity, as can be seen from the contact angle values before and after the modification.

4.3. Repeated immobilization of bioreporters and their visualization in SEM

By measuring surface charges and zeta potentials of used microorganism, authors previously showed that the surface modification with APTES will lead to increase of quartz surface hydrophobicity and enhanced adsorption of *P. putida* TVA8. The same approach was used to immobilize strain 652T7.

OFE modification with APTES leads to successful formation of TVA8 biofilm layer on its surface (Fig. 6). Nevertheless, this approach was unsuccessful with the 652T7 strain. Visible TVA8 biofilm developed on all five available OFEs. Two days after beginning of the bioluminescence inductions, lumps of cell colonies (100-1000 µm apart) between much smaller scattered clusters or single cells were observed on the glass surface under SEM, which corresponds to standard biofilm establishment characteristics (15). The cell layer kept developing until it covered the entire OFE face surface, as it could be seen from the SEM sample that was visualized after 130 days of repeated inductions (Fig 6).

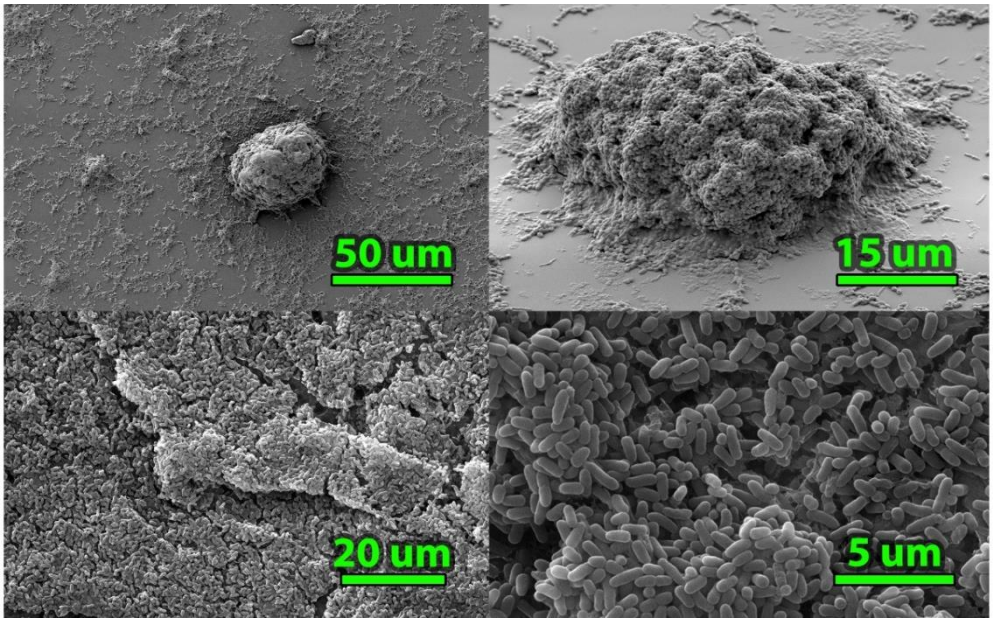

Fig. 6: P. Putida TVA8 biofilm-like layer, island colonies and scattered cells across OFE surface, 2 days after initial immobilization (top). P. Putida TVA8 biofilm layer, covered surface, 130 days after initial immobilization (bottom). (17)

4.4. Time records of induced bioluminescence

Typical time-records of daily inductions of bioluminescence of *P. putida* TVA8, such as records from bioreporters immobilized on OFE "0", are shown in Fig. 7. Y-axis denotes detected bioluminescence intensity in photon detector counts per second (denoted as A.U.). X-axis denotes time from the induction of bioluminescence in hours. Shown experiment lasted 26 days. Chart legends denote the measurement day number.

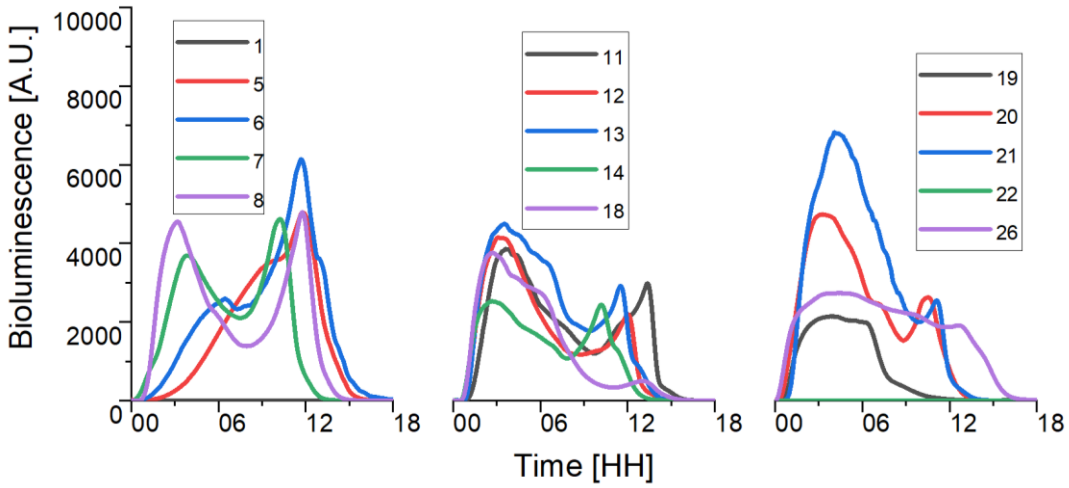

Fig. 7: OFE – 0 - X axis: time (HH); Y axis: bioluminescence (A.U.), legend shows day number

Successful colonization of the OFEs surface was proved by induced bioluminescence. Results among the repeats of the trial varied significantly, nevertheless, these common features were observed:

1) Two bioluminescence maxima were observed on the daily records of induced bioluminescence (Figs. 7-8). The first peaks B1 were caused by adsorbed biofilm cells. The second peaks B2, which appeared 8–12 h after B1, were the results of cells growth that were often released from the adsorbed layer into the solution.

Small second peaks confirmed experimental calculations, which suggested the best place to immobilize bioreporters is on the very surface of OFEs wider end. The size or presence of the second peak depended on the distance of the OFE from the bottom of beaker with induction solution, and thus on the varying amount of induction solution with different number of bioreporter cells underneath the OFE face (see beaker on adjustable stand in Fig. 3). This distance changed every time the induction solution was changed, and the stand with

the baker was set again. The size and nature of the B2 make it an insignificant element for the purpose of toluene detection.

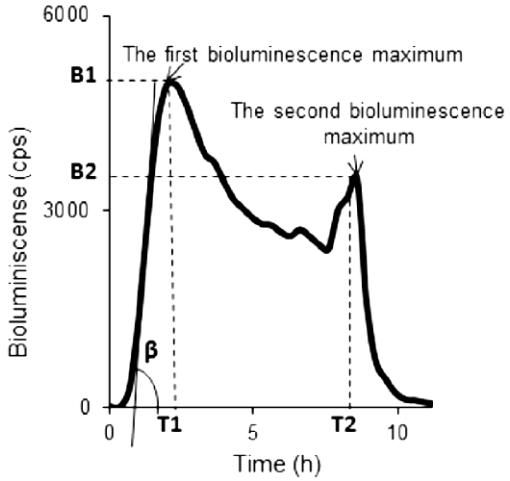

Fig. 8: Typical time-record (smoothed) of daily induction of P. putida TVA8 adsorbed on the wider end of tapered quartz optical fiber modified with APTES. The inductor (toluene 26.5 mg L−1) was added at time = 0.

2) During the first several days of the experiment, the bioluminescence maxima of the principal peak were gradually developing (Fig. 9) and were achieved in shorter times (Fig. 10). Times of appearance of the P1 maxima decreased within first 5 days and was between 2 – 4 h after the induction, and stabilized at about 4 h 46 min (Fig. 10). Nevertheless, any signal above twice the amount of background noise of the detector (Oriel module - 2×0.26 nA; Perkin Elmer module 2x20 cps integrated over 10s) could reliably confirm the presence of toluene in the sample. Such an increase in bioluminescence appeared within 0.5 h after immersion in the induction solution. This growth was observed in all inductions (for all OFEs) with exceptions over the first two days. During this initial period, the cell layers were likely not matured and performed slowly, with low bioluminescent responses.

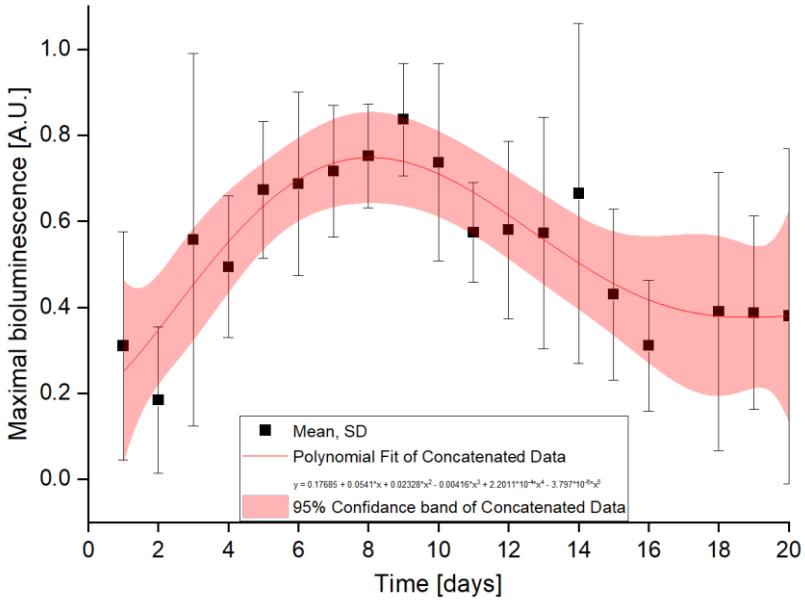

Fig. 9: Daily bioluminescence maxima (B1) normalized to 1. Aggregated data from six OFEs.

3) During the second week of the experiments all the lines showed two peaks. The intensities of the detected light (B1) were low during the first few days after induction and then gradually increased (Fig. 9). This might be ascribed to an advanced covering of the base of the OFE with cells. Light intensities (B1) decreased after 8-14 days and were fluctuating across a wide range (Fig. 9). Number of leaked cells increased over time, resulting in higher bioluminescence intensities from the solution (B2).

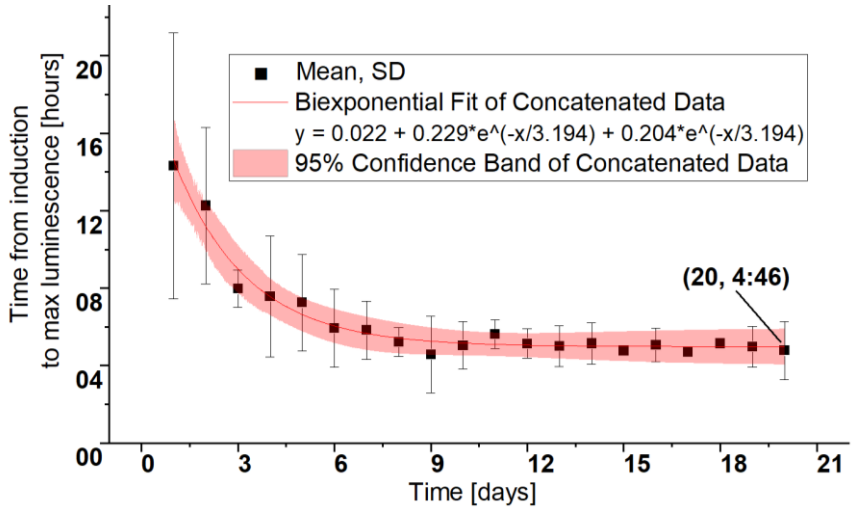

Fig. 10: Time of the first bioluminescence maxima; aggregated data of six OFEs.

4.5. ICP experiment

The experiment carried out at ICP was aimed to test the longevity of inductions. The cells adsorbed on the element were induced 68 times with toluene and 4 times with contaminated ground water over the course of 135 days.

The sum of emitted light (S), was tested, comprising the contributions of light emitted from both the adsorbed and leaked cells and the rate by which the bioluminescence increased (β). The bioluminescence maxima (B1, B2) and the times at which they were achieved (T1, T2) from the longest trial with the element.

While the bioluminescence maxima (B1, B2) fluctuated across a large range (Fig. 9), the times of appearance of the first bioluminescence maxima (T1) were between 2 h and 4 h (Fig. 10) and for T2 were between 10 h and 14 h. The courses and fluctuations of S and β were similar to the fluctuations of B1 or B2. The reproducibility of all the analytical responses tested (B1, B2, T1, T2, S, β) remains so low that the element with adsorbed cells is plausible only for identification of the presence of bioavailable BTEX. The fiber biosensor with adsorbed bioluminescent bioreporters was repetitively induced with toluene for more than 2 months, which is the longest time-interval reported for the service of such a biosensor. The technique of cell adsorption and the conditions of bioluminescence inductions require optimization to achieve better reproducibility. (48) (49)

4.5.1. Contaminated water induced bioluminescence

The biofilm-like layer on OFE was induced with “Aqua43” 60 days after immobilization. At that time, the bioluminescence responses to the induction solution were lower than during the first 30 days. Nevertheless, the intensities of bioluminescence induced with this contaminated water were much lower than expected based on the content of toluene (172 mg L^{-1}) and were also lower than the intensities induced with the induction solution (26.5 mg L^{-1}). This decrease in induced bioluminescence probably results from a toxic effect of high concentrations of toluene and xylenes and increased temperature above 30 °C on the days of measurement. On day 71, the ten-times diluted “Aqua43” did not increase the bioluminescence of adsorbed cells. Nevertheless, the following day-72, the maximum

of the bioluminescence response was higher. This one-day delay in the increase in bioluminescence maxima might be a lag-time needed for cells adaptation.

On day 78 the element was immersed in a growth medium with the aim to support the viability of adsorbed cells. The following day the intensity of bioluminescence induced with the induction solution reached 4900 counts. Despite this restoration of light production, the bioluminescence responses decreased over the following days. After 135 days the presence of the bioluminescent bioreporter *P. putida* TVA8 on the element was confirmed by induction with a toluene solution containing kanamycin, which inhibits the growth of bacteria other than TVA8, and detection of bioluminescence.

The study demonstrated the repetitive inductions of bioluminescence of the adsorbed cells of a bioluminescent bioreporter. The cells were adsorbed on an APTES-modified tapered optical fiber element. The adsorbed cells performed a two-fold higher number of repetitive inductions than cells entrapped in silica gel. The element is conceivable as a detection probe for multiple use in a laboratory and the online monitoring of bioavailable contamination in remote localities.

4.6. UTK experiment

The experiment at UTK was aimed to test the repeatability of inductions and to compare different OFE geometries. The cells adsorbed on 5 different elements over the course of 2-3 weeks. The cells adsorbed on the element were induced 10-16 times with toluene over the period of 20 days. Besides the B1 bioluminescence maxima, the sum of emitted light (integral of bioluminescence peak) was also tested. B1 maxima and the sum of its bioluminescence correlated, as it could be seen from the Fig 11.

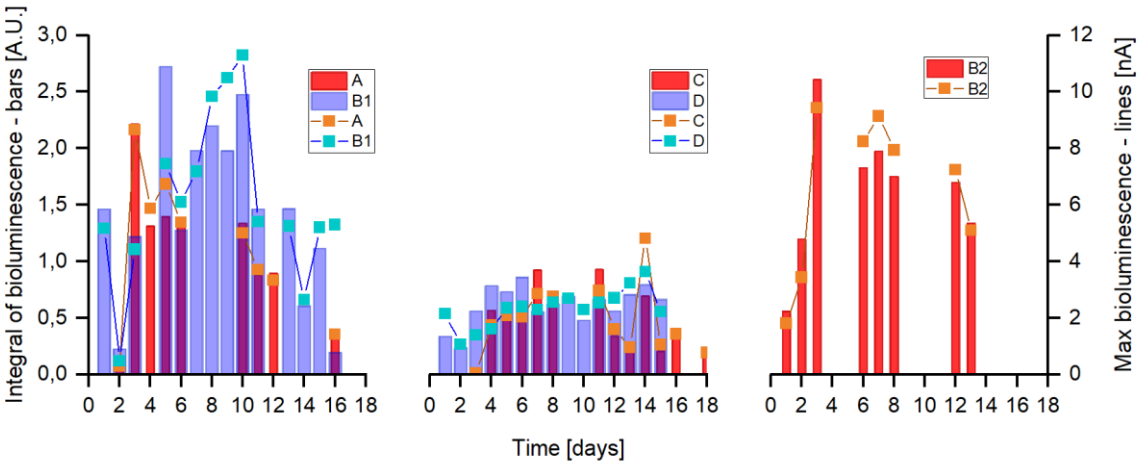

Fig. 11: Main peak bioluminescence integrals and main peak max bioluminescence of each OFE within 20 days.

Table 6 compares time-records, bioluminescence maxima, integrals of bioluminescence, and OFE efficiencies. Unbiased OFE efficiency parameter suggested the highest bioluminescence signal should be detected with the OFE "D". Our calculations did not fully match the real measurements. Transmittance differences of tenths of a percent did not result in a significant difference of detected bioluminescence.

Table 6: Sums of bioluminescence intensities and maxima of bioluminescence for five OFEs

OFE	BL sum of light, average value [A.U.]	BL sum of light, max value [A.U.]	BL average value [nA]	BL max value [nA]	OFE efficiency x 10^5
A	1.1056044	2.2179	4.4899	8.65	3.48
B1	1.4585701	2.7255	6.1605	11.30	2.72
B2	1.6217301	2.6090	6.5433	9.44	3.71
C	0.4597357	0.9305	1.8573	4.82	1.38
D	0.6002724	0.8607	2.2790	3.63	11.75
Ranks from the highest to the lowest					
	B2>B1>A>D>C	B1>B2>A>C>D	B2>B1>A>D>C	B1>B2>A>C>D	D>B2>A>B1>C

These results confirmed previous observations that OFEs adhering with *P. putida* TVA8 can be repeatedly used as a detector for toluene after a few days of the stabilization of immobilized cells. A stabilization period of two to four days was observed, even if the cells were immobilized in silica gel. (12) (34)

Kalabova et al. (2018) was able to predict a different outcome of experiments comparing an OFE and a PCS fiber, but in authors experimental results the effect of used bioreporter cells surpassed the differences in OFE transmittances. Used mathematical model is useful for the design and better understanding OFEs in terms of coupling and transmitting bioluminescence to a detector, the best shape, size, and ideal place for bioreporter immobilization. (36)

Possible ways to improve the stability of bioluminescence signal responses include engineering a cell strain with two reporter genes (one under control of an analyte of interest and one constitutively present to monitor cell viability). An alternative to this is the use of two independent bioreporters (constitutive and inducible) derived from the same strain. Nevertheless, in the optical fiber arrangement, this resolution requires two fibers, which complicates the sensor construction. The analyte-specific signal must be than corrected according to cell viability (50); or genetical manipulation of a bacterial strains ability to create and dissolve biofilm structure (51) (52).

4.6.1. Immobilization and induction of *E. coli* 652T7

In the LB_{Kan} growth medium, *E. coli* 652T7 did not adhere on the APTES modified base of the OFEs regardless of the addition of ferric chloride which was added to theoretically enhance the adhesion of microorganisms by lowering the repulsion forces (53). Cell attachment was observed only at the interface of growth medium and air (Fig. 12). At this interface, photon-OFE binding efficiency is < 1%, thus bioluminescence of these attached cells would not significantly contribute to detected light.

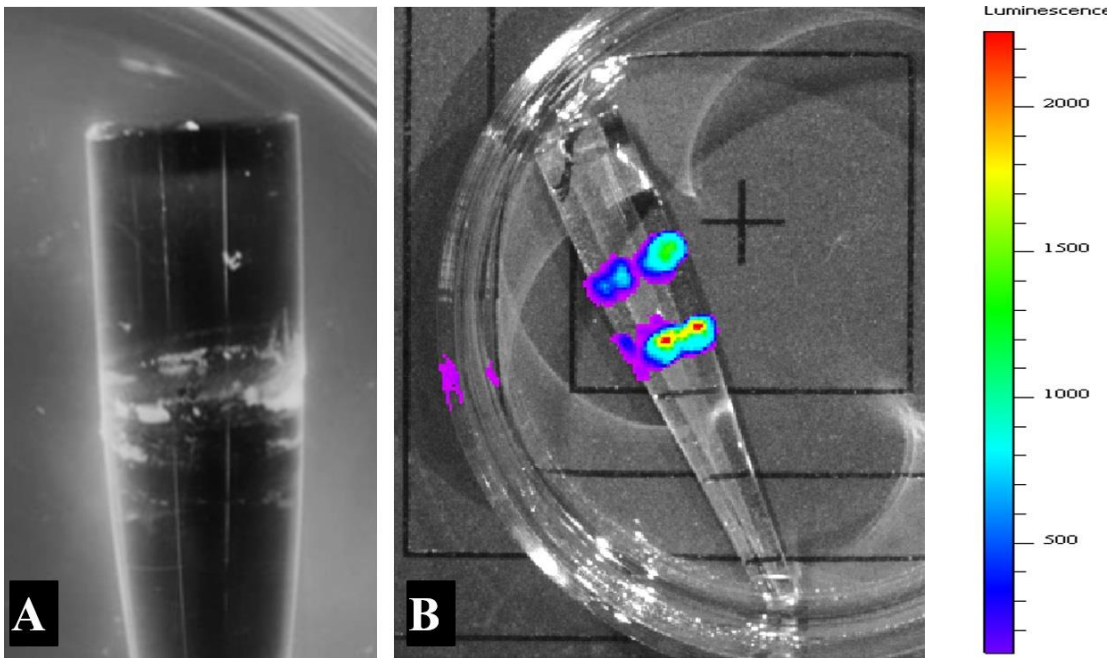

Fig. 12: E. coli 652T7 *on the surface of an APTES modified quartz cone after 4 days in* LB_{Kan} *cultivation medium (A). Bioluminescence of E. coli 652T7 induced after immersion in LB medium as measured in an IVIS Lumina K imager (photons/sec/cm*^{2}*/steradian) (B).*

E. coli 652T7 was immobilized on the base of OFE-D with PEI. The time records of daily inductions with LB medium is presented in Fig. 13. Except for the first induction intensities, bioluminescence increased within 15 min after immersing the OFE into the LB solution. The intensities remained stable for 18 h on the first day, 6-9 h on all other days, and then sharply decreased due to a depletion of nutrients. To test the OFE with immobilized *E. coli* 652T7 as a biosensor for biotoxicity, HCl was added to the induction solution on the 8th day. This caused pH lowering to pH = 6 and decreased the bioluminescence, which did not recover after the following two inductions. These results imply that an OFE immobilized with *E. coli* 652T7 is sensitive to influences that affect cell viability but cannot be repetitively used as a biosensor since cells are dying and not recovering.

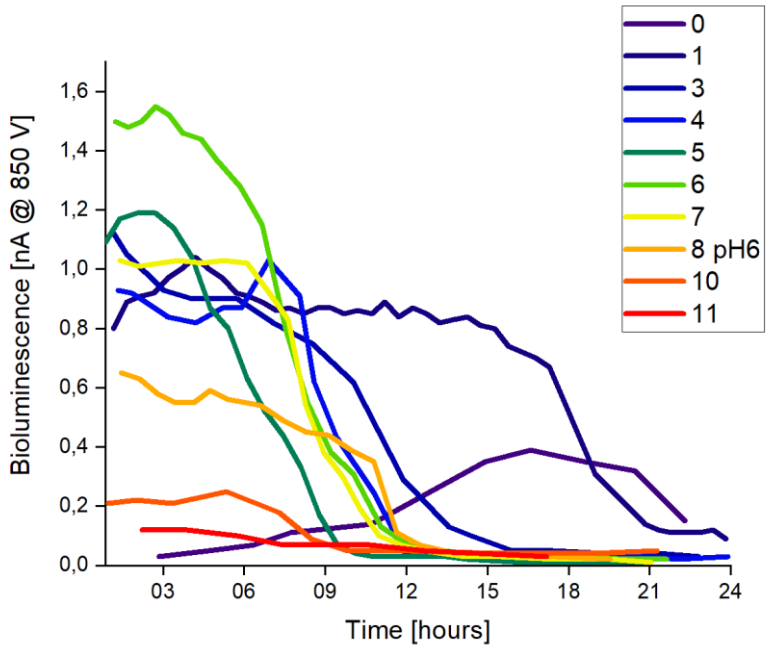

Fig. 13: Time records of bioluminescence of E. coli 652T7 immobilized on OFE D in PEI. Legend denotes the days after the immobilization.

In the UTK study we immobilized the bioluminescent bioreporter *P. putida* TVA8 on a tapered OFE in order to prepare a biosensor for the detection of liquid toluene. This study broadened our previous research where we used physico-chemical models, using contact angles and zeta potential, to facilitate the attachment of *P. putida* TVA8 to quartz surfaces after treatment with APTES. Biofilm development of *P. putida* TVA8 with time was quantified and the repeatability of the biofilm preparation and the repeatability of bioluminescence detection was determined. Except for a short maturation period (~2-4 days), the OFEs exhibited a stable bioluminescent response for at least 20 days.

We additionally immobilized the constitutively bioluminescent toxicity bioreporter *E. coli* 652T7 on a PEI modified OFE and demonstrated its potential use as a biosensor for cytotoxicity. Also, the immobilization process that we used, without any bulky matrix requirements, could be applied towards many other microbial bioreporters for the biosensing of a variety of different analytes. However, since the reproducibility of bioreporter responses remains low, the developed biosensor can be used for online, rapid and multiplexed monitoring of the presence of a pollutant, but not its concentration.

5. Conclusions

The author made a literature research on pollution, effect of pollutants on human health, current detection methods of toluene, and the areas where is the principle of bio-sensing used. Author also become familiarized with the work and procedures used in a microbiological laboratory.

The biosensor for the long-term detection of liquid toluene in a laboratory sample was developed. The author used physico-chemical models, using contact angles and zeta potential, to facilitate the attachment of bioluminescent bioreporter *P. putida* TVA8 to quartz surfaces of tapered OFEs after its treatment with APTES. Bioreporter cells were successfully immobilized without an inorganic carrier in a biofilm layer. The best shape of an OFE was proved to be a Frustum cone. The biofilm development of *P. putida* TVA8 with time was quantified and the repeatability of the biofilm preparation and the repeatability of bioluminescence detection was determined. Other than a short maturation period (~5 days), the OFEs exhibited a bioluminescent response for the period of 135 days. Sensitivity of the developed sensor was also successfully tested on a real polluted water sample.

It was demonstrated that constitutively bioluminescent toxicity bioreporter *Escherichia coli* 652T7, immobilized on a PEI modified fiber element, could potentially serve as a biosensor for cytotoxicity. Additionally, the used immobilization process used, without any bulky matrix requirements, could be applied towards many other microbial bioreporters for the biosensing of a variety of different analytes. However, since the reproducibility of the bioreporter responses remains low, the developed biosensor can be used for online, rapid and multiplexed monitoring of the presence of a pollutant, but not its concentration.

Cooperation between teams from the Faculty of Biomedical Engineering of the Czech Technical University, the Institute of Chemical Processes of the Czech Academy of Sciences, and the Center for Environmental Biotechnology of the University of Tennessee resulted in publication of two articles in impacted journals, and three international conference contributions.

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7. List of works related to the dissertation

7.1. Impacted journal scientific papers

• **Repetitive inductions of bioluminescence of *Pseudomonas putida* TVA8 immobilised by adsorption on optical fibre**. Zajíc, J., Bittner, M., Brányik, T., Solovyev, A., Šabata, S., Kuncová, G., & Pospíšilová, M.; *Chemical Papers* 2016, 70(7), 877-887; IF_{2016} = 1.258; doi: 10.1515/chempap-2016-0031

• **Repetitive Detection of Aromatic Hydrocarbon Contaminants with Bioluminescent Bioreporters Attached on Tapered Optical Fiber Elements**. Zajíc, J.; Ripp, S.; Trögl, J.; Kuncová, G.; Pospíšilová, M.; *Sensors* 2020, 20, 3237; IF_{2019} = 3.275; doi: 10.3390/s20113237

7.2. Conference active participations and proceedings

• **Whole-cell detectors of contaminants constructed by immobilization of bioreporters in form of biofilm on special optical fiber elements.** Zajíc, J.; Ripp, S.; Trögl, J.; Kuncová, G.; Pospíšilová, M.; *IEEE International Conference on Sensors and Nanotechnology*, Penang, Malaysia, 2019, pp. 1-4; doi: 10.1109/SENSORSNANO44414.2019.8940080.

• **Whole-Cell Detectors of Aromatic Hydrocarbon Contaminants Constructed by Immobilization of Bioreporters on Special Optical Fiber Elements.** Zajíc, J.; Ripp, S.; Trögl, J.; Kuncová, G.; Pospíšilová, M.; Morava J.; *Instruments and Methods for Biology and Medicine*, 10th Student Scientific Conference, Kladno, Czech Republic. ISBN: 9788001067963.