



Biosensor Chip for Monitoring of Water Pollution as Novel Test Tool in Public Health: Proof of Principle

Nadira Ibrišimović Mehmedinović, Aldina Kesić, Almir Šestan, Aida Crnkić, Mirza Ibrišimović

Abstract: Humans are generally exposed to a variety of pollutions present in the air they breathe, the food they eat or in the water they drink. Some of the most dangerous pollutions are metals and heavy metals. These are naturally occurring substances which are harmless when present in the environment at low levels. However, due to many pollutants such as industry processes or war activities, the heavy metal concentration can exceed the limit of tolerance and become very toxic for the natural environment and living organisms in it, including humans. Unlike organic pollutants, the heavy metals (as ions and as particulate matter) once introduced into the environment cannot be biodegraded and remain there indefinitely. By rainfall these pollutants can be partially transferred from air or soil into the rivers and drinking water sources, where they accumulate in even higher toxic levels. The high concentrations of heavy metals in contaminated natural water reservoirs have an impact on the microbial community composition which resides there. This type of water pollution can cause the changes in life cycles of natural bacterial populations, influencing their metabolic processes and proliferation. The presence of pathogens in water is normally indirectly determined by the testing for "indicator organism" such as coliform bacteria. Coliforms are usually present in larger numbers in contaminated water and at the same time they are indicators of whether other pathogenic bacteria are present, too. In crisis situations, like war or some natural disasters, where trusted sources of drinking water are not available anymore, the military and residents of affected areas are forced to use some alternative water resources that cannot be tested for their microbial or metal contamination properly. Therefore, the existence of some fast test that would detect not only dangerous bacterial pathogens in water, but also the presence of metals and heavy metals as well, would be of great help and importance for the human health. Even though the number of pathogens can be drastically reduced by the boiling of water, the heavy metals are not destroyed by high temperature. Hence the main objective of our work was to optimize the biosensor chip for microbial detection in contaminated water that would serve at the same time as an indicator for the chemical composition of the water,

such as presence of metals and heavy metals, with potential to be used as a novel test tool in public health.

Keywords: Biosensor, Heavy Metals, Microbes, Water Pollutants.

I. INTRODUCTION

Routine control of microbiological food and water quality include both the total number of microorganisms and the presence of pathogens. Even non-pathogens, if present in large enough numbers, can cause deterioration of food products and distaste in water (1). Coliform bacteria can serve as indicators of fecal contamination (2). Coliforms are defined as "Gram-negative aerobic or facultative anaerobes, nonspore-forming, rod shaped bacteria that ferment lactose with acid and gas production. The coliform group includes the genera of *Esherichia*, *Enterobacter*, *Klebsiella* and *Citrobacter* (3).

Many metals serve as cofactors for enzymes which are excreted by bacteria, e.g. coliforms, which are commonly present in polluted water. Based on the fact that the biosensor technology that was used in this study is capable of detecting microbial enzymes as described in some our previous studies (4-7), manifestation and intensity of the signal was changing in dependence on which metal/heavy metal is present in a test sample. It was shown that presence of some metals in water, like zinc, manganese or calcium which are building up the active site of some microbial enzymes, can increase the sensitivity and functionality of the biosensor. On the other hand, some heavy metals like mercury can inhibit the metabolic activity of microorganisms and lower down the biosensor's detection signal. Water contamination can include some radioactive elements like cesium and polonium which are generally released from explosives, and which are found in natural waters worldwide (8).

Heavy metals are found naturally in the earth and are not treating to the environment if present in low trace. However, if their concentrations exceed the limit of tolerance, they can become very toxic to the natural environment and living organisms in it, including humans (9). Metals can be introduced in aquatic systems by weathering of soils or from different human factors including the war activities, the mining or some other industrial processes.

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* Correspondence Author

Nadira Ibrišimović Mehmedinović*, Department of Chemistry, Faculty of Natural Sciences and Mathematics, University of Tuzla, Tuzla, Bosnia and Herzegovina. Email: nadira.ibrisimovic@untz.ba

Aldina Kesić, Department of Chemistry, Faculty of Natural Sciences and Mathematics, University of Tuzla, Tuzla, Bosnia and Herzegovina. Email: aldina.kesic@untz.ba

Almir Šestan, Department of Chemistry, Faculty of Natural Sciences and Mathematics, University of Tuzla, Tuzla, Bosnia and Herzegovina. Email: almir.sestan@untz.ba

Aida Crnkić, Department of Chemistry, Faculty of Natural Sciences and Mathematics, University of Tuzla, Tuzla, Bosnia and Herzegovina. Email: aida.crnkic@untz.ba

Mirza Ibrišimović, Gynecological polyclinic "Korak do života", Tuzla, Bosnia and Herzegovina and Sarajevo Medical School, University Sarajevo School of Science and Technology, Sarajevo, Bosnia and Herzegovina. Email: ibrisimovic2001@yahoo.com

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Unlike organic pollutants, the heavy metals once introduced into the environment cannot be biodegraded and remain there indefinitely (10, 11). Methods for remediation of heavy metals from contaminated environment are very complicated and time demanding, including physical removal, bioleaching and detoxification (11). In water, when the pH decreases, metal solubility and toxicity will increase and the metal particles can become more mobile. Since the habitats for aquatic organisms are usually the water or sediments, the captured heavy metals can accumulate in tissues of species residing there, including fish and macrophytes. Besides water, the heavy metals like zinc, cadmium, mercury, and chromium can pollute the air and soil, and contaminate the living forms present there. The sensitivity of individuals of a particular aquatic species to heavy metals may be influenced by factors such as age, sex, or size. The accumulation of heavy metals in plant or animal tissues can influence their life cycle and reproduction and through the food chain endanger the human health (12, 13).

For example, the concentration of metals in invertebrates is conversely related to their body mass. It is expected that the embryonic and larva stage of fish is the most sensitive to this type of pollution (12). Some of the water bacteria are capable of converting mercury to the toxic methyl mercury that accumulate in fish and can cause serious health problems for the human body (13). A long-term exposure to heavy metals can cause an acute or chronic damage of nervous system, renal dysfunction, lung disease and lately there is an increasing number of reports about stimulated effect of heavy metals on some types of human cancer (15-17).

The metabolic features of microorganisms (primarily bacteria), which were closely investigated in course of our study, were used to optimize the biosensor matrix, the biosensor's setup, and the type of color change that is displayed as an optical detection signal. This biosensor technology enabled identification of the aquatic ecosystems in Bosnia and Herzegovina containing toxic amounts of heavy metals, which can endanger living organisms habiting there and human health as well. The study was proof of principal of optimized patented biotechnology chip (7) as a fast test for presence of microbiological and chemical contaminants in water.

II. MATERIALS AND METHODS

A. Collection of Water Samples

For our work we used the water samples of the largest rivers in Bosnia and Hercegovina: Bosna, Drina, Sava and Neretva. From all rivers the samples were taken in urban areas with approximately the same number of inhabitants per m². After the water samples were taken in 1000 mL plastic bottles, they were analyze for their chemical and microbial status. First parameters being analyzed were: electrical conductivity, pH and temperature.

B. Determination of Heavy Metals Amount Using AES

The collected water samples were analyzed in laboratory for trace metals by Atomic Emission Spectrometer (AES). The following heavy metals were analyzed: zinc (Zn), lead (Pb), chromium (Cr), iron (Fe), manganese (Mn), barium (Ba), tin (Tin), thallium (Tl) and selenium (Se). The detection of traced metals was performed by AAS. It is known that heavy metals regularly form complexes with organic constituents. Therefore, it was important to digest the organic

components by using a strong acid that would in contrary cause interference and an unreliable measuring value. Interfering were removed by using 37 % hydrochloric acid (HCl)- end concentration of 1% in water samples.

C. Microbiological Analysis of Water Samples and Recognition of Indicators for Microbial Water Quality

For the identifications of microorganisms we used ÖNOZ and Aero Pseudo Selective Agar (Himedia), and other conventional microbiological methods (MacConkey agar, Manitol salt agar from Merck).

D. Quantitative Monitoring of Microbial Enzymatic Activity

For testing of enzymatic activity we determined Leucine aminopeptidase (LAP) which is a common virulence factor of coliform bacteria such as *Enterobacter spp.*, *Esherichia spp.* and *Pseudomonas spp.* A test samples was examined for the exopeptidase LAP activity that selectively releases N-terminal amino acid residues from polypeptides and proteins. Since the temperature plays very important role in activation of the enzyme, the enzyme dilutions must be incubated for 15 minutes at 37°C. A calibration line can be generated by means of increasing LAP activities. Therefore a stock of LAP with a specific activity of 10 U/mg must be prepared. The used LAP dilutions should be in the range of 10 to 1000 mU/mg. The reagents which will be used are:

a) 60 mM Phosphate Buffer, pH 7.2 at 37 °C (Prepare 100 ml in deionized water using potassium phosphate, monobasic, anhydrous. Adjust to pH 7.2 at 37 °C with 1 M KOH.).

b) 1.66 mM L-Leucine p-Nitroanilide Solution (L-Leu_NA) (Prepare 30 ml in Reagent a using L-leucine p-nitroanilide hydrochloride. Prepare fresh!).

c) 10 mM TrisHCl Buffer with 1 mM MgCl₂, pH 8.0 at 37 °C (Activation Buffer) (Prepare 20 ml deionized water using Trizma Base. Adjust to pH 8.0 at 37 °C with 1 M HCl and then add MgCl₂ x 6H₂O).

d) Leucine Aminopeptidase Enzyme Solution, Non-Activated (Enz-Non Act) (Immediately before use, prepare a solution containing 0.3 unit/ml of leucine aminopeptidase in cold deionized water).

e) Leucine Aminopeptidase Enzyme Solution, Activated (Enz-Act); (Immediately before use, prepare a solution containing 0.1 U/ml of leucine aminopeptidase in cold Reagent c. Incubate at 37 °C for 15 minutes to activate.

E. Development of the Polymer-Based Biosensor Chip

The presented biosensor setup consists of a reflective layer made up of e.g. Ni-Cr alloy which is covered with a polymer layer (substrate). The polymer layer is normally made up of PLGA (poly lactic-co-glycolic acid) dissolved in ethyl acetate containing varying amounts of desmodur.

Desmodur serves as a linker of the PLGA molecules influencing the viscosity and composition of the polymer, which can be additionally doped with various compounds mediating specific effects.

PLGA was approved in the year 2000 by the FDA (Food and Drug Administration) for *in vivo* use and applications in drug targeting.

When specific lytic enzymes such as those secreted by bacteria come into contact with the polymer layer (specific substrate), the polymer is topically degraded, leading to a color change due to altered refraction of visible light. The biosensor specifically identifies secreted lytic enzymes and therefore offers the possibility of detecting bacterial metabolic activity even in absence of the causative pathogen in the specimen analyzed. Microbes in total count of 10 000 CFU/ml in liquid, overnight culture, were incubated on the biosensor for different incubation times, and presence of metabolic activity of the microbes was detected due to color change of the biosensor.

III. RESULTS

A. In Situ Chemical Analysis of Water Samples and Determination of Heavy Metals Amount Using AES

All used water samples were analyzed with Hanna HI-9829 Multiparameter pH/ISE/EC/DO/Turbidity. Results of in situ measurement are listed in tables 1 and 2. *In situ* measurement indicated existence of differences in analyzed parameters for all river samples, showing especially high values for water samples from river Bosna.

Elevated levels of TDS for river Bosnia can be connected to the fact that this river and its tributaries are passing through places with heavy industrial activities, which are main cause for environmental pollution.

Measured concentrations of heavy metals are listed in table 3. As we can see in table 3 the highest values in heavy metal concentration, from all nine heavy metals being analyzed, showed iron and strontium. The concentration of iron in river Sava was from 0,126 mg/mL and for strontium in concentration from 0,167 mg/mL.

Table 1. In situ measurements.

River	Temp. [°C]	pH	mV [pH]	ORP [mV]	EC [μS/cm]	EC Abs. [μS/cm]
Bosna	13,56	6,51	5,8	151,2	412	401
Drina	13,45	7,64	-56,6	140,2	209	203
Sava	13,90	7,82	-66,2	128,5	218	214
Neretva	9,33	7,66	-43,1	182,9	283	199

ORP-oxidation reduction potential

EC- electrical conductivity

Table 2. Results of chemical analysis of water samples.

River	RES [Ohm-cm]	TDS [ppm]	Sal. [psu]	D.O. [%]	D.O. [ppm]
Bosna	2427	206	0,20	72,9	6,04
Drina	4785	104	9,82E-02	72,7	6,04
Sava	4587	109	0,10	67,2	5,54
Neretva	3500	141	0,14	91,4	10,37

TDS- Total dissolved solids

D.O. – Dissolved Oxygen

Table 3. Concentrations of heavy metals in mg/mL from water samples from rivers Bosna, Drina, Sava, and Neretva.

River	Heavy metals concentration (mg/mL)				
	Ba	Cr	Fe	Mn	Pb
Bosna	0,008	0,004	0,087	0,029	0
Drina	0,002	0,006	0,085	0,033	0,005
Sava	0,005	0,004	0,126	0,022	0,006
Neretva	0,001	0,001	0,000	0,003	0,000

River	Heavy metals concentration (mg/mL)			
	Se	Sn	Sr	Tl
Bosna	0,032	0,035	0,110	0,037
Drina	0,034	0,038	0,085	0,024
Sava	0,023	0,032	0,134	0,015
Neretva	0,036	0,036	0,167	0,045

B. Identification of Microorganisms in Water Samples

Using conventional microbiological identification methods, from tested water samples it was possible to identify the following microorganisms as habitants in rivers Bosna, Drina, Sava and Neretva: *Enterobacter spp.*, *Esherichia spp.*, *Pseudomonas spp.* and *Proteus spp.*. *Enterobacter spp.*, *Esherichia spp.* and *Pseudomonas spp.* were found in every tested water sample.

C. Monitoring of Microbial Metabolic Activity by Leucine Aminopeptidase (LAP) Detection in Water Samples

For monitoring of microbial growth and metabolic activity testing, we used isolated strains of *Enterobacter spp.* and *Esherichia spp.*, which were inoculated in tryptic soy broth for 24 hours at 37°C and their metabolic activity was measured after different incubation times (after 0h, 16h, 24h, 32h and 48h). Since the iron and strontium as representatives of heavy metals were the most present in tested water samples, they were chosen as substrates for addition to *Enterobacter spp.*, *Esherichia spp.* and *Pseudomonas spp.* growing cultures, after which LAP activity was detected.

LAP serves as virulent factor for these microbes and levels of its secretion were not constant in *in vitro* conditions, after various incubation times (figures 1-3). In order to monitor the impact of heavy metals, we used heavy metals Fe and Sr in two concentrations, as follows: Fe1 = 0.085 mg / L; Fe2 = 0.126 mg / L; Sr1 = 0.085 mg / L and Sr2 = 0.167 mg / L. As we can see from the figures 1-4, the presence of heavy metals had a stimulating effect on the virulence of microorganisms, and elevated excretion of LAP could be observed.

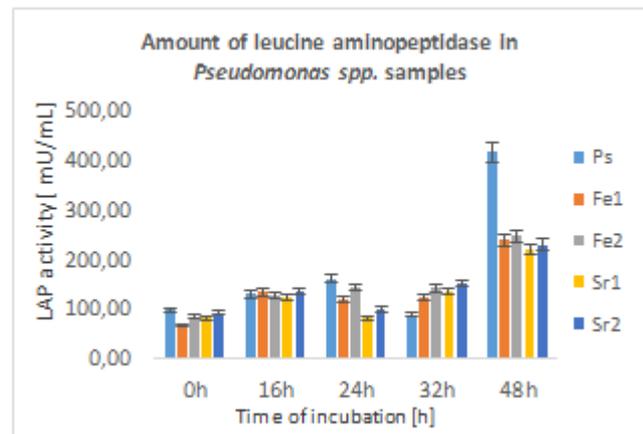


Figure 1. Influence of heavy metals iron (Fe) and strontium (Sr) in samples of Pseudomonas spp.

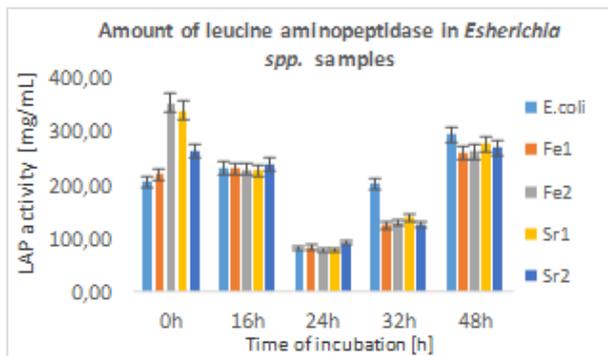


Figure 2. Influence of heavy metals iron (Fe) and strontium (Sr) in samples of *E. coli*.

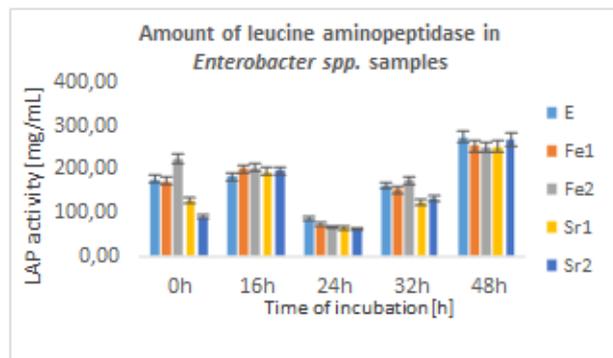


Figure 3. Influence of heavy metals iron Fe and strontium Sr in samples of *Enterobacter* spp.

D. Proof of principle for biosensor chip detecting heavy metal pollutants and microbial contaminants in water ecosystems

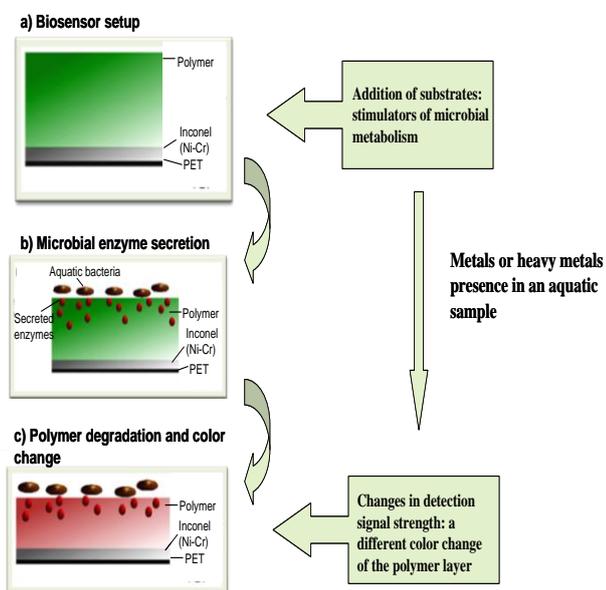


Figure 4. A polymer-based, optical biosensor chip for detection of microbial and chemical contamination in an aquatic sample and its mechanism of action.

When lytic enzymes (e.g. secreted by bacteria) come into contact with the polymer layer of biosensor, the polymer will be degraded resulting in a topical color change of the biochip, for example from green to red (figure 4).

Some of the additives act as a carbon and/or energy source for specific microorganisms and provide the nutritional

environment triggering the release of lytic enzymes, thus improving the efficacy of detection.

For functionality test we used biosensors with PLGA concentration in range between 18-23% PLGA with 1% Desmodur and substrate glucose, tryptone and inorganic salts. All the biosensors were incubated by 37°C for 4h and 24h.

Proof of principle of biosensor chip for monitoring of water pollution are carried out with microorganisms and water samples. (Ps- *Pseudomonas* spp., Ec- *E. coli* spp., En- *Enterobacter* spp.)

The color changes shown in figure 5 and figure 6 are result of enzymatic degradation of the biomimetic polymer layer by lytic enzymes. The degradation is highly selective and clearly visible with naked eyes. The signal intensity correlates with increasing incubation time.

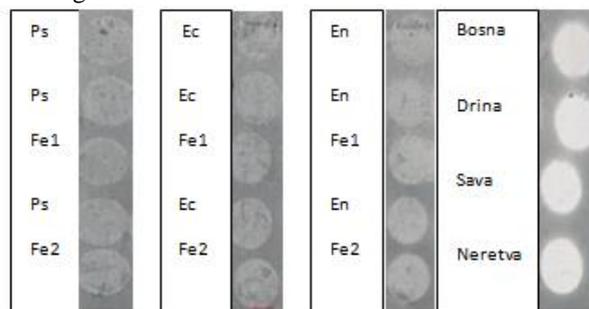


Figure 5. Signal intensity after incubation for 4 h and temperature at 37°C.

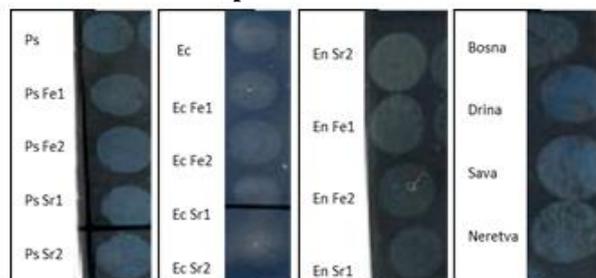


Figure 6. Signal intensity after incubation for 24 h and temperature at 37°C.

IV. CONCLUSIONS

Coliform bacteria are routinely used as an indicator of the microbiological quality of water. They are present in the environment and feces of all warm-blooded animals and humans.

The coliforms are unlikely to cause illness, but their presence in water reservoirs indicates that disease-causing organisms (pathogens) could be in the water system. Since the testing of water samples of different origin, for all possible pathogens is complex, time-consuming, and expensive, this problem is solved by testing for coliforms only, applying simple and inexpensive methodology, such as biosensor chip technology presented in our study. In our study we were also able to show that here presented patented biosensor technology can be optimized for detection of bacterial contamination in water and joined with heavy metal contamination.



Experiments with iron and strontium for strains *Esherichia spp.*, *Pseudomonas spp.* and *Enterobacter spp.* showed that signal intensity on surface of biosensor is increasing due to addition of heavy metals as substrates, in comparison to bacteria alone. This means that presented biosensor chip can be used as dual detecting system for detection of bacteria, parallel with heavy metals, as commonly present pollutants in water ecosystems, and be used as a novel testing tool in protection of public health.

The water samples of the rivers Bosna, Drina, Sava and Neretva showed a clearly visible signal on the surface of the used biosensors that is visible to the naked eye, and based on these results we can conclude that the optimized design of biosensors can detect water pollution in the field in a very short time. The population of risk areas would be able to identify non-drinking water, because its consumption could be harmful to their health.

Since the optimized biosensor can detect metals and heavy metals in all types of accumulated water (groundwater, rivers, lakes, drinking water reservoirs, etc.), it can also be used as a rapid detection method for industrial wastewater, which generally contains a high level of heavy metal.

Due to the fact that most of the aquatic ecosystems we examined are geographically related to other aquatic systems (rivers) in Europe, the obtained data on chemical contamination and its impact on aquatic flora and fauna, including human health, is very important scientific contribution to European water systems as well.

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AUTHORS PROFILE



Nadira Ibrišimović Mehmedinović, is associate professor at Department of Chemistry, Faculty of Natural Sciences and Mathematics, University of Tuzla, Tuzla, Bosnia and Herzegovina. Nadira Ibrišimović Mehmedinović is author of 23 scientific publications and four books, including two patents: 1. Ibrišimović, N., Pittner, F., Ibrišimović, M., Barth, M., Bohrn, U. (2009). Device comprising a polymer layer and a reflecting

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Aldina Kesić, is associate professor at Faculty of Natural Science and Mathematics, at University in Tuzla, Bosnia and Herzegovina. Aldina Kesić is author of 42 scientific publications and seven books and book chapters. She was a mentor for the preparation of 6 master's theses. She is the head of the chemistry department and the president of the Tuzla Canton Association of Chemists. Selected publications:

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Almir Šestan, is associate professor at Department of Chemistry, Faculty of Natural Sciences and Mathematics, University of Tuzla, Tuzla, Bosnia and Herzegovina. Almir Šestan is author of 28 scientific publications. His research area is in general and inorganic Chemistry.

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Aida Crnkić, is professor at Department of Chemistry, Faculty of Natural Sciences and Mathematics, University of Tuzla, Tuzla, Bosnia and Herzegovina. Her research is in the field of food chemistry, environmental chemistry, and chemistry of medicinal plants.

Aida Crnkić is author of 42 scientific publications.

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Mirza Ibrišimović, is associate professor at Sarajevo Medical School, at University Sarajevo School of Science and Technology in Sarajevo, Bosnia and Herzegovina, and also works as embryologist and cytoscreener-supervisor in Gynecology Polyclinic "Korak do života" in Tuzla, Bosnia and Herzegovina. Mirza Ibrišimović is author of 22 scientific publications and four books, including one patent: Patent: Ibrišimović, N., Pittner, F., Ibrišimović, M., Barth, M., Bohr, U.

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