

## ANNOTATION

### Of the material under Art. 65 of the Regulations for the development of academic staff of PU "Paisii Hilendarski"

#### Of chief asst Mariana Ivanova-Marhova-Koseva, PhD

For participation in a competition for the academic post of Associate Professor in the field of higher education 4. Natural sciences, mathematics and informatics, professional field 4.3 Biological sciences (Microbiology - Microbial pathogenesis), Announced in State Gazette 92/18.11.2022.

For participation in the competition for the academic position of "Associate Professor" I submit an author's monograph and 35 scientific publications that were not used for the acquisition of the PhD and the academic position of "Chief Assistant Professor". The scientific works are distributed as follows:

- ❖ Monograph "Drug resistance and virulence in *Enterobacteriales* uropathogens. Studies from 1997 to 2021."
- ❖ 15 publications in journals with impact factor or impact rank, which are refereed and indexed in world-renowned databases with scientific information - Web of Science and Scopus;
- ❖ 15 publications in refereed journals without impact factor or impact rank;
- ❖ 5 publications in proceedings of scientific conferences with scientific review;
- ❖ 1 co-authored textbook;
- ❖ 1 co-authored manuscript.

The monograph and the scientific papers are the result of research in two main areas:

- ❖ **Microbial pathogenesis.**
- ❖ **Ecology of microorganisms.**

The teaching aids are related to my participation in the educational process at the Faculty of Biology of PU "Paisii Hilendarski"

#### **I. Annotation of materials in the field of microbial pathogenesis**

Urinary tract infections (UTIs) are one of the most commonly diagnosed infections in humans and represent a serious health and economic problem for society. The treatment of these diseases is related to the correct diagnosis of the etiological agents, the identification of drug susceptibility and virulence characteristics important for success in the pathogenetic process. Monitoring drug resistance of microorganisms is not only important in selecting appropriate therapy, but is also imperative because of the remarkable ability of microorganisms to acquire resistance through multiple mechanisms. The increasing antibiotic resistance of microorganisms is a global environmental problem and necessitates the search for alternative approaches to the therapy of bacterial infections.

The area "Microbial pathogenesis" includes the materials presented in the monograph and publications No. **2.1; 2.2; 2.3; 2.4; 2.5; 3.1; 3.2; 3.5; 3.7; 3.8; 3.11; 3.13; 3.14; 3.15; 4.1, 4.2 and 4.5**

In the presented monograph "Drug resistance and virulence of uropathogens from *Enterobacterales*. Studies in the period 1997 - 2021" includes results of own studies and summaries on *Enterobacterales* uropathogens conducted in the period 1997 - 2021. An overview is given of the revolutionary advances in molecular biological analysis methods since the late 20th century and their application in metagenomic analyses and bacterial identification. *Enterobacterales* are a remarkable group of microorganisms with a wide distribution. They form part of the normal gut microbiota in humans and a range of animals, most of the time in commensal relationships with their hosts. Possessing chromosomally encoded virulence determinants or acquiring such determinants in plasmids, transposons, integrons and other mobile genetic elements, members of the order can cause a wide range of diseases.

The studies of isolates from samples of outpatients with infections of the urogenital tract, conducted in the period 1997-2021, were the first of their kind in volume and duration for the region of Plovdiv and southern Bulgaria. Strains isolated during microbiological examination of urine and genital swab samples provided by outpatients with uncomplicated and complicated UTI were studied and/or documented. The samples were initially examined in the microbiological laboratory of the Medical University - Plovdiv, the microbiological laboratory of the Military Medical Academy - Plovdiv (Military Hospital), the Chronolab - Plovdiv, the Zinvest-K - Plovdiv (2012.), MDL "Cibalab" - Sofia (2014), "Sinevo" EAAD - Plovdiv (2016-2017 and 2021) and constitute a representative sample of outpatient visits for diagnosis, therapy and prevention of diseases of the urogenital tract. The data from the surveys were analyzed and compared with the publicly announced information from NCPB and BulStar. The etiological map of urogenital tract infections in outpatients was compiled and *Escherichia coli* emerged as the predominant etiological agent in the urogenital tract: 72.74% of the 157 strains studied in the period 1997-2012, 74% of positive urine samples issued by GB SMDL Zinvest - K Ltd. for 2012; 66% of positive urine samples issued by MDL Cibalab Ltd. for 2014; 55% of positive urine samples and 23% of positive genital swab samples issued by Sinevo - Plovdiv for the period April 2016 - March 2017 and 54% for 2021. For the period 1997 - 2021, *Klebsiella* spp., *Proteus* spp., *Enterobacter cloacae*, *Staphylococcus* spp., *Enterococcus* spp. and to a lesser extent *Candida* spp. dominated genital swabs. Over the period, variability in the proportion of *Enterobacterales* species in the UTI was reported. The relevance of the monitoring carried out is strongly confirmed taking into account the incomplete and insufficiently detailed information disclosed by NCIPD and BulStar. The need for coordinated action by diagnostic laboratories and regulatory authorities to disclose such data is clearly outlined in order to reduce the incidence of an empirical approach to the treatment of UTIs.

During the period from 1997 to the present day, clinical laboratories in Bulgaria have applied a well-established protocol proposed in the 1960s, which provides for aerobic cultivation of a small amount of a mid-morning urine sample on a limited set of media for 18-24 h. Parameters that favour *E. coli* and *Enterobacteriaceae* but leave no chance of identifying slower growing species with more distinct culture preferences. For the period 1997 - 2021, 17.15% (BulStar 2001,) 24.32% (GBMDL Zinvest K, 2012), 24.10% (MDL Cibalab, 2014), 27.9% (Sinevo for 2016-2017) and 33.64% (Sinevo, 2021)

of samples were reported as positive or "with growth". An alarmingly high percentage of samples are reported as negative or "no growth". Considering the statistics on the prevalence of UTIs, we believe that many more cases remain unreported and not well enough diagnosed to be adequately treated. Our study strongly points to the need to intensify the accumulation and sharing of data and results at the national level in order to develop and validate an expanded culture protocol for microbiological examination of urogenital tract specimens to increase reliability in the diagnosis of UTI.

Systematized data on antibiotic susceptibility of urinary isolates from outpatient samples were compared with those reported by BulStar, taking into account that in the majority of the publicly available dataset there is no distinction between outpatients and inpatients. NCIPD and BulStar also reported increasing resistance to cephalosporin drugs as well as increasing ESBL isolates. High levels of antibiotic resistance and the disparities found at regional and national levels strongly reject the empiric treatment of UTI in the community. Differences in resistance levels by region necessitate the development of an antibiotic resistance monitoring system at both regional and national levels through the improvement of the National BulStar Programme.

Studies and analyses for the period 1997-2021 have shown an increase in quinolone resistance and a sustained prevalence of strains with an ESBL phenotype. Initial studies have shown the presence of plasmid-mediated quinolone resistance, but further analyses are needed to confirm it. The dynamically changing levels of resistance found once again confirm the need for continuous local updating of medical practices and approaches to UTI therapy.

Uropathogenic *E. coli* (UPEC) express chromosomally and plasmid-encoded virulence determinants. Studies of the virulence characteristics of *Enterobacterales* uropathogens have demonstrated the "many" faces of urovirulence by strongly confirming the importance of the adhesive potential of strains for success in colonization of the urinary tract and biofilm formation as a defense strategy.

New knowledge about the normal microbiota in the urinary tract, as well as more extensive case series research, indicates that it is likely that a large proportion of UTIs are multispecies and that the cumulative resistance is a derived variable dependent on many factors: characteristics of the polymicrobial species involved, relative quantitative species involvement, and host immune status. It seems appropriate to track the characteristics of *in vitro* cultured biofilms involving two or more strains in future studies. Knowledge of the composition of the normal urobiota, application of modern metagenomic and metatranscriptomic analyses to investigate the spatial and chemical basis of interspecies relationships and their impact on the host will illuminate potential synergistic relationships leading to success in pathogenesis.

The emergence and spread of drug resistance among bacterial pathogens is one of the most significant problems for modern medicine. The possibility of adaptive resistance to amikacin in *Pseudomonas aeruginosa* is documented in **Publication 3.1**. A strain of *P. aeruginosa* isolated from an infected wound was used. The minimum inhibitory concentration of MIC<sub>Amikacin</sub> was determined and the emergence of resistant

mutants was monitored under selective amikacin pressure at concentrations higher than the MIC. Within 120 h, mutants with altered resistance were isolated, as shown by the significantly higher MIC reported, from 20 to more than 70 µg/ml. Higher resistance to complement action was reported for the isolated mutants.

**Publication 3.2** presents the results of a study of the sensitivity of 54 uropathogenic *E. coli* strains to complement action in native human serum. Nine strains (16.7%) proved to be fully resistant, and thirty (55.6%) showed intermediate resistance. The plasmid content was examined and some of the strains were "cured" of them. This made it possible to account for the involvement of genetic determinants of plasmid composition in serum resistance. For some of the plasmid-free derivatives, an increase in sensitivity to complement action was found, whereas the plasmid-free derivative of *E. coli* strain 77 retained intermediate resistance. The results of this study provide evidence for the involvement of plasmid-encoded determinants in the serum resistance of uropathogenic *E. coli*.

Uropathogenic isolates with established serum resistance were further subjected to serological and cytochemical analysis. The studies are presented in **publication 2.1**. Rabbit hyperimmune sera were obtained from ten selected strains with a significant difference in serum sensitivity in a 4-step immunization schedule with 3- or 4-day intervals. The similarity in antigenic determinants of the selected strains was monitored by conducting agglutination on glass, in a test tube and cross-absorption. Two pairs of strains selected on the basis of similarity in serological characteristics and serum sensitivity were used to monitor concanavalin A - gold binding and affinity in the native state and after heat treatment at 100°C for 10'. The results showed differences in localization, density and accessibility of Con A binding epitopes in the serologically similar pairs of strains. Additionally, a direct correlation between previously tested serum sensitivity and the density of Con A binding epitopes was demonstrated. In the more resistant *E. coli* 73 and 88 strains, serum resistance appeared to be associated with membrane-associated epitopes.

**Publication 3.5** presents the study of 20 isolate strains from samples of patients with chronic UTI. Biochemical tests identified thirteen of the strains as *E. coli*, three as *Pantoea agglomerans* (*Enterobacter agglomerans*), three as *Citrobacter freundii*, and one as *Citrobacter diversus*. Antibiotic resistance study showed high sensitivity to aminoglycoside preparations and high resistance to ampicillin, doxycycline, clindamycin. Three of the *Citrobacter* spp. strains showed multiple resistance. Six of the strains (30%) were found to produce broad-spectrum β-lactamases (ESBL). Plasmids ranging in size from 3 to 10 kb were detected in eight of the strains tested.

In **publication 2.2**, the results of the study of 28 *E. coli* strains isolated from samples of outpatients with various UTIs are reported. Thirteen strains (46.43%) were found to be multidrug resistant and six (21.43%) produced ESBLs most likely of the STX-M type. Plasmid content analysis showed the presence of plasmids ranging in size from 2 kb to over 23,000 kb. High resistance to quinolone preparations and sulfonamides was found. The strains showed good sensitivity to nitrofurantoin.

In **Publication 2.3**, the ability of 50 uropathogenic *E. coli* strains to form biofilm *in vitro* was investigated. Antibiotic susceptibility, morphotype, production of type 1 pili, haemolysins were investigated. Biofilm-forming strains proved to be a significant fraction, 36% of the strains studied. Data showed no significant correlation between combinations of virulence factors studied and biofilm formation.

In **publication 3.7**, a contemporary topic related to established intercellular communication in microbial communities, the "quorum sensing", is commented. This process enables bacterial populations to exhibit multicellular behaviors in response to community density and composition. Many pathogenic microorganisms regulate the expression of their virulence characteristics using quorum sensing. This makes signaling pathways an attractive target for the development of inhibitor molecules to interfere with the infectious cycle. Potential strategies to interfere with quorum signaling are discussed in this publication.

**Publication 2.4 and Publication 2.5** explore alternative approaches to antibiotics for influencing and modulating the characteristics of uropathogenic *E. coli*.

In **Publication 2.4**, the effects of medicinal plant extracts against three uropathogenic strains with good biofilm-forming capacity were investigated. The extracts did not exhibit antibacterial activity but showed modulating biofilm-forming capacity that varied widely. In order to fully characterize the potential of the tested extracts, the active constituents need to be specified.

In **Publication 2.5**, the modulatory effect of dilute cell-free culture supernatants (CFS) of *Lactobacillus* spp. with probiotic potential was investigated: six strains of lactic acid products and five vaginal isolates. Changes in properties such as hydrophobicity and motility, biofilm formation of reference and uropathogenic strains were investigated. The exopolysaccharides present in CFS stimulated biofilm formation, but proteinase K reduced the inhibitory activities of the supernatants. This suggests the involvement of a protein factor in the suppression of biofilm formation.

In **publication 3.8**, the antimicrobial activities of new derivatives of benzimidazole as an alternative to antibiotics are investigated. Antimicrobial activity was demonstrated for eight of the synthesized compounds against Gram-positive *Staphylococcus aureus*, *Enterobacter aerogenes* and antifungal activity against *Candida albicans*.

**Publication 3.11** documents the representation of virulence characteristics and biofilm-forming capacity in twenty uropathogenic *Escherichia coli* strains. Multiplex PCR reactions were performed and the presence of genetic determinants *fimH* for type 1 pili, for RAR pili, capsular antigens for capsules K1 (group II), K5 (group II) and K5 (group III); for toxins *cnf1* and *hlyA*, for serum resistance - *traT* and *iss* was documented. Detailed analysis of the virulence characteristics examined did not reveal a dominant pathotype, but there is no doubt that type 1 pili are a universal tool for bacterial pathogenesis in the urinary tract.

**Publication 3.13** presents the study of antibiotic and serum resistance of 318 strains of the family *Enterobacteriaceae* isolated from infections in the urogenital tract. *E. coli* was the most frequent etiological agent of UTI in outpatients, 64.8%, followed by

*Klebsiella spp.* (17%) and *Proteus mirabilis* (10.37%). High resistance to ampicillin (49%), mecillin (71%), doxycycline (41%) was found. At the same time, high susceptibility was found to cephalosporins (cefuroxime - 84.6%; cefoxitin 83.7%; cefotaxime 91.5%; cefepime 83%) and fluoroquinolones (ciprofloxacin 85%, norfloxacin 79%, levofloxacin 83%). Significant resistance was reported suspended nitrofurantoin (24%). Among the strains studied, 8.5% produced broad-spectrum ESBLs. 84% of the strains were found to be resistant to the lytic action of normal human serum, demonstrating the importance of this virulence characteristic for successful pathogenesis in the urogenital tract. The results of this study demonstrate the need for continuous monitoring of drug sensitivity to avoid empiric therapy.

In **Publication 3.14**, the antibiotic resistance of 72 *Enterococcus faecalis* strains from samples of outpatients with urogenital tract infections was monitored. Additionally, virulence characteristics such as enzyme production and biofilm formation were investigated. Enterococci were found to be more frequently isolated from samples of women. While enterococcus-associated UTIs were more common in children under 10 years, genital tract infections were more common in women of reproductive age. High (30%) resistance to gentamicin has been found. Relatively low (19%) resistance to fluoroquinolones with clear cross-resistance was found in isolates from UTI. *E. faecalis* isolates from genital tract samples showed higher (22%) resistance to norfloxacin and low levels of resistance to levofloxacin (4%) and ciprofloxacin (2%). Among the strains isolated from the urinary tract, 37% produced gelatinase and 93% produced caseinase. In genital swab isolates, these values were 27% and 67% for gelatinase and caseinase, respectively. Of the 72 strains studied, 26% formed biofilm *in vitro* with the majority of biofilm-forming isolates (70%) from genital swabs. The hydrolase activity found in most of the enterococci studied proves its importance as a virulence factor in the colonization of the urogenital tract.

**Publication 3.15** presents the results of studies on resistance and virulence factors in 97 *Candida* strains isolated from urine and genital swabs of outpatients. Among the isolates, *C. albicans* (84%), followed by *C. glabrata* (7%), *C. krusei* (4%), *C. parapsilosis* (3%) and *C. tropicalis* (2%). The overall sensitivity to the antifungal agents tested was high at over 95%. Analysis of hydrolytic enzyme activity and biofilm forming ability showed that only 8% of the strains produced gelatinase and phospholipase, 6% produced caseinase and 5% esterase. Seven of the strains tested formed a stable biofilm after 24 hours of cultivation in Sabouraud broth enriched with 6% glucose. No significant correlation was found between antifungal susceptibility and the virulence factors tested. The strains studied had rather commensal characteristics, suggesting that fungal infections in the urogenital tract infections studied are more often the result of dysbiosis and/or a change in the balance of the normal microbiota.

**Publication 4.5** is a review of experience in bacterial and helminth research with lectin-based methods. The cell surface of all organisms is characterized by the presence of glycoconjugates (glycoproteins and glycolipids). They are important players in intercellular interactions, including those between infectious agents and their hosts. Glycoconjugates are a major class of pathogen-associated molecular structures that

interact with host molecules recognizing foreign antigens. One major class of recognition molecules are the carbohydrate-binding proteins, lectins, widely distributed in viruses, bacteria, and all plant and animal taxa. In turn, many lectins with known carbohydrate specificity are included in methodologies for the analysis of cellular glycoconjugates. In genetically identical bacteria, large differences have been found between individual cells with respect to the lectin-binding epitopes accessible on the surface. Within bacterial populations, the balance between different cells varies depending on culture conditions, which also leads to variations in the ability of host lectins to recognize them. Individual variations in the composition of surface sugars have been linked to evolutionarily determined changes in the infectivity and virulence of pathogens in different hosts. The phenomenon of surface carbohydrate variation is ubiquitous in pro- and eukaryotic pathogens and this demonstrates its importance in pathogen-host relationships.

## **II. Annotation of materials in the field of ecology of microorganisms**

The area "**Ecology of microorganisms**" includes materials divided into two groups :

### **❖ Publications Nos. 2.6; 2.7; 2.8; 2.10; 2.12; 2.14; 3.10; 4.4;**

The research presented in these publications is related to:

- Investigation of the microbiological status and sanitary condition of several of the major dams in Bulgaria - reservoir. Kardzhali, Dospat Dam, Batak, Tsankov Kamak (**NºNº 2.6; 2.7; 2.8; 2.12; 3.10; 4.4**)
- Ecosystem assessment of protected wetlands in southern Bulgaria (**2.10; 2.14**)
- ❖ Publications Nos. 2.9; 2.11; 2.13; 2.15; 3.3; 3.4; 3.6; 3.9; 3.12; 4.3;**
- studies of enzyme production and optimization of cultivation of strains with potential for application in bioremediation and biotechnological production (**NºNº 2.9; 2.13; 3.3; 3.4; 3.6; 3.9; 3.12; 4.3**)
- research on the condition and quality of soils in urban environments and the creation of sustainable models for bioremediation and soil management (**NºNº 2.11; 2.15**)

In Bulgaria, in recent years there has been an increased interest in cage culture as an industrial form of aquaculture in inland freshwater ponds. Due to increased investor interest, the number of new cage farms is also increasing. The volume of consumption of raw fish products (hot- and cold-smoked) is increasing, raising human health concerns. Fish and fish products are thought to be vectors of bacterial infections associated with both natural inhabitants of aquatic ecosystems and those introduced into them as a result of fecal contamination of human and animal origin. At the time of our research in Bulgaria, there were no data on the impact of aquaculture on the bacteriological status of the hydro ecosystems of the water bodies in which they are located. Furthermore, in recent decades there has been increased urbanization and development of coastal areas, which increases the risk of contamination by domestic sewage. This calls for increased attention to sanitary parameters of water quality, including assessment of microbiological contamination (total number of microorganisms) and sanitary status (total coliform count) of waters used for aquaculture. Publications **3.10; 4.4; 2.6; 2.12** consistently present

data on biological indicators and ecological parameters of waters and sediments in the reservoir area. Kardzhali for potential assessment of its aquaculture capacity.

**Publication 4.4** presents a microbiological study of the water quality in the Kardzhali reservoir in April-August 2011 by determining TVC<sub>37°C</sub> and total coliform count (TC) at a total of six stations in the reservoir (one near the dam, three around the dam farms and two in the dam-free part of the reservoir). TVC<sub>37°C</sub> values ranged from 70 CFU/100 ml in April to 251. 10<sup>3</sup> CFU/100 ml in the summer period. TVC<sub>37°C</sub> and TC values were found to be several times elevated in the waters of the Arda River compared to the waters of the Kardzhali Reservoir. These are likely to have influenced the higher values recorded in St. VI, which is in the flow zone of the reservoir, where the influence of the river Arda is felt. The obtained results indicate additional organic pollution in the studied area. The increased number of coliforms in the net-cage area coincides with the intensive tourist season in the nearby settlement of Glavatartsi, which lacks an established sewage return system. This creates preconditions for filtering of contaminated water from the septic facilities of the hotels into the waters of the reservoir Kardzhali. The sharp increase in coli-titer values from 1 bacterial cell in over 100 ml of water sample in spring to 1 CFU/2.5 ml in August is a clear indicator of faecal contamination. The values reported for the indicators in the period April-August 2011 indicate additional organic contamination in the areas around the cages in Kardzhali Reservoir.

**Publication 3.10** includes data on the main microbial indicators of water quality and microbiological status of Kardzhali Reservoir for a one-year period from April 2011 to March 2012. Indicators monitored include total *E. coli*, faecal streptococci (FS) and *Clostridium perfringens* counts at six stations in the reservoir and one in the Arda River. With the exception of August and October, *E. coli* and FS values did not exceed 30 CFU/ml and 20 CFU/ml, respectively. In August, an elevation of both indicators was detected, which persisted until November. Statistically significant differences in both indicators were found in the stations around the cages and in the area of Glavatartsi settlement, areas with strong anthropogenic influence. The presence of *Clostridium perfringens* was recorded only in August 2011 in the areas around the cages and the dam wall. To establish the composition of the microbiota within the dam, 220 faecal coliform strains were isolated and identified. All isolates belonged to five genera of the family *Enterobacteriaceae*. *Klebsiella* (39%), *Enterobacter* (24%) *Serratia* (25%), *Citrobacter* (7%) genera were found to be the most abundant in the reservoir waters, respectively represented by species *K. oxytoca* and *K. pneumoniae*, *C. amalonaticus* and *C. freundii*, *E. cloacae* and *E. aerogenes*, *Serratia marcescens*. High levels of sewage contamination, based on coli-titer and coli-index studies (200 cfu *E. coli*/1 l) were found in the waters of the Arda River in the section before it flows into the Kardzhali Dam. The absence of pathogenic species combined with the decrease in TC, coli-titer and coli-index at station II is evidence of the self-cleaning capacity of the water body studied. The new increase in the number of TC at station I near the village of Glavatartsi, as well as the presence of faecal coliforms, indicates secondary sewage contamination in the area. The observed microbiological parameters show a pronounced seasonal dynamics in the Kardzhali dam and the Arda river, with peak values of all indices in August. The observed species



composition of the coliform group gives grounds to assume that microbiological pollution is mainly due to increased environmental pressure caused by human activities in the area.

In **publication 2.6.** the studies of antibiotic resistance of bacterial isolates from sediments in the water area of the Kardzhali reservoir are presented. Antimicrobial resistance is a direct result of the selective pressures caused by the overuse of antibiotics, including the misuse of antibiotics in veterinary medicine and aquaculture. The study included 160 strains of Gram-negative bacteria isolated from sediment samples from station 1 near the net cages and station 2 at the top of the dam, which is cell-free and used as a control station. The susceptibility to 16 antimicrobials was reported for 100 strains of *Pseudomonas mandelii*, 30 strains of *Hafnia alvei*, and 30 strains of *Raoultella ornithinolytica*. There were no significant differences in resistance to the antibiotics tested between the strains isolated from the two stations (analysis of variance,  $P > 0.05$ ). However, high resistance to penicillins, amoxicillin and erythromycin was reported. *P. mandelii* strains exhibited resistance to tetracycline, which is thought to pose a significant risk for dissemination. Relatively low resistance to quinolones was reported. No resistance was reported to the aminoglycosides gentamicin and amikacin or to ciprofloxacin. *Pseudomonas* isolates showed the highest rates of antibiotic resistance and the highest MIC values. A large proportion of the strains tested, 62%, showed resistance to chloramphenicol, which is one of the most commonly used drugs in aquaculture and banned for use in the European Union due to its proven cytotoxicity. The results of the study showed a high frequency of the presence of resistant Gram-negative bacteria in the sediments below the cages in the lake Kardzhali.

**Publication 2.12.** documents studies conducted in the Kardzhali reservoir to establish the relationship between abiotic environmental factors, microbiological indicators of water quality and phytoplankton with respect to the development of intensive cage aquaculture. A multivariate analysis was performed based on data for eighteen indicators from 5 monitoring sites obtained in the period 2016-2018. The objective was to identify key parameters affecting biological communities, including the impact of nursery farms. ANOSIM (analysis of similarities), showed significant differences in the values of physicochemical factors between the control site and the aquaculture area, with higher nitrate, total nitrogen and COD (chemical oxygen demand) near the nets. The results were confirmed by the high R-value ( $R = 0.87$ ;  $p < 0.01$ ). The conducted PCA (principal component analysis) showed that the physicochemical parameters could be grouped into only three principal components (PCs) - PC1 was formed by the forms of nitrogen and COD, PC2 reflected the physical source of variation (pH and dissolved oxygen), and PC3 was formed by total phosphorus (0.537) and ammonium nitrogen (0.764). The three components account for 90.5% of the total variance. Parameters with the greatest impact on heterotrophic bacterial abundance (TVC) included temperature, TN, and COD. Phosphorus was not a limiting factor due to its high concentration. The multivariate analysis (RDA) clearly showed that in dams with intensive cage aquaculture, long-term farm operation is associated with local changes (limited to the vicinity of the farms) in water physicochemical parameters, resulting in quantitative and qualitative changes in phytoplankton and bacterial communities.

An analysis of the waters of the reservoir Dospat is presented in **publication 2.7.**, carried out in order to establish the microbiological status of the water body and to identify possible secondary sources of pollution in the aquatorium. Six stations located in the reservoir water area and one station on the Dospatska River were selected for the assessment of the main microbiological indicators. The seasonal and spatial dynamics of total microorganisms (TVC 20°C), total coliforms, *E. coli*, faecal streptococci and *C. perfringens* for the period April 2011 - March 2012. Values for TVC 20°C appeared to range from  $1.10^3$  cfu/100 ml to  $39.10^3$  cfu/100 ml, with no statistically significant differences between stations. Values for total coliforms ranged from 10 cfu/100 ml to 100 cfu/100 ml, and increased to 1000 cfu/100 ml in August, with higher levels near the farm. Coliforms were characterized by low species diversity and dominance of *Serratia marcescens*, *Pantoea agglomerans*, *Hafnia alvei* and *Enterobacter cloacae*, which are part of the natural microbiota of water bodies. The analysis of TVC 20°C and total coliforms showed a pronounced seasonal dynamics in the Dospat reservoir, and the waters of the Dospatska River in the study period were characterized by higher levels of microbial load compared to the open water area of the water body. The high values of the indicators near the city of Sarnitsa are indicative of the discharge of fecal-domestic wastewater. Their secondary rise near the net-cages is evidence of significant anthropogenic pressure in the area.

In **Publication 2.8** a comprehensive metagenome analysis of the planktonic bacterial community of two large and economically important dams in Bulgaria - Batak Dam and Tsankov Kamak Dam - is presented for the first time. Microorganisms inhabiting freshwater environments are an integral part of the aquatic ecosystem. Currently, there is very little data on the taxonomic composition of microbial communities in Bulgaria's dams, despite their key role in biogeochemical processes. Analysis of metagenomic sequencing data showed that 78.45% of the microbiome overlapped between the two dams. Diversity (H) and community evenness (J) indices decreased along the longitudinal axis of the two dams. The values found for the Shannon diversity index are more characteristic of oligotrophic water bodies. The dominant complex in both reservoirs is formed by the *Proteobacteria* division, followed by *Actinobacteria* and *Bacteroidetes*, which account for over 95% of the relative abundance, despite large hydrogeological differences. Bacterioplankton are characterized by high phylogenetic heterogeneity in taxonomic structure. The presence of 211 genera was found. The genera *Limnohabitans* and *Rhodospirillum rubrum* are dominant, suggesting their importance in aquatic food webs. The data obtained will contribute to a better understanding of microbial biodiversity in freshwater environments and serve as a basis for future comparisons and analyses.

Another direction of research in the field of microbial ecology is related to the characterization of microbial communities in wetlands in southern Bulgaria (**Publication 2.10.**) and in the Maritza River basin (**Publication 2.14.**). Wetlands are important ecological areas or aquatic areas where microbial communities play an essential role in primary productivity, nutrient cycling and water purification. Constructed wetlands are a low-cost ecological approach based on the ability to remove anthropogenic pollution through biological methods. Wetlands are located in areas of low elevation and play an

important role in purification near urban areas. Research on interspecific communities and their ability to develop metabolic networks and biofilms may be useful in bioremediation processes of contaminated habitats.

**Publication 2.10** presents the analysis of microbial communities and their biofilm-forming capacity in two Natura 2000 protected wetlands - Zlato Pole and the rice fields near Tsalapitsa in southern Bulgaria. The numbers of heterotrophic bacteria (TVC 22 and TVC 37), actinomycetes, fungi, and sanitary microorganisms in dry soil and sediment samples were determined. The number of heterotrophic microorganisms and sanitary status indicators (FS, FC, and *Escherichia coli*) in the two rice belts near Plovdiv city were higher compared to the control zone in Zlato Pole; the maximum was recorded in the rice field near Tsalapitsa village ( $C_1$  and  $C_2$  was  $12.6 \times 10^6 \text{cfu.g}^{-1}$  and  $26 \times 10^6 \text{cfu.g}^{-1}$ , respectively). In the samples studied, the bacterial complex occupied a dominant position and exceeded the number of mould fungi and actinomycetes at least 1.5 times. Cluster analysis showed a high similarity between soils surrounding rice fields and sediments from Gold Field (ZP<sub>2</sub>) due to the low organic load. The biofilm-forming capacity of the multispecies assemblages was investigated in four different *in vitro* culture media. The highest values were recorded in R2A medium for all stations. Our results from the *in vitro* biofilm-forming study showed a good correlation between microbial community structure and biofilm-forming capacity.

**Publication 2.14** includes our research on spatial variation and physiological diversity of soil microbial communities in natural wetlands and constructed rice fields in the Maritza River basin, protected under the Birds Directive 2009/147/EC as natural habitats. The wetlands located along the Bulgarian part of the Maritza River basin are part of the Ramsar Convention and represent rare specific ecosystems. The metabolic functionalities of the communities in the studied wetlands vary considerably, demonstrating that their profile is shaped by a wide range of environmental factors such as soil water content, pH, organic matter and nitrogen sources. PCA and cluster analysis show that long-term agricultural exploitation alters soil properties and bacterial communities, grouping rice fields into a distinct cluster. Continuous rice cultivation in the wetlands of Tsalapitsa resulted in soil acidification and higher total metabolic activity, but lower catabolic and substrate diversity, making microbial communities sensitive to stress and external factors. The higher substrate diversity along with the sandy, river-like sediments, low concentrations of organic nitrogen, organic matter and phosphate are evidence of the good ecological potential of the Golden Field wetland. This is confirmed by the higher metabolic activity in terms of hard-to-digest polyols and amino acids due to the absence of easily digestible carbohydrates. A detailed analysis of bacterial diversity in two different wetlands in the Maritza River basin is presented. The results reveal a significant difference in bacterial community structure between the permanently flooded sediments of the Golden Field wetland and the seasonally flooded sediments of the Ricefields protected area near Tsalapitsa. Multivariate analyses grouped the soil microbiome in the Maritza River basin based on soil type, wetland type, and reclamation activities carried out in the wetland. The results confirm the significant influence of

environmental factors on the structure of microbial communities, which are affected by anthropogenic pressures.

**Publication 3.3** provides an overview of the potential of microbial biotransformations in bioremediation. Knowledge of the abilities of microorganisms to transform different xenobiotics creates a good basis for the development of effective technologies for remediation of contaminated sites. Currently, the search and study of unusual metabolic pathways for the uptake of various xenobiotics and previously considered non-degradable substances is extremely relevant.

**Publication 3.6** reports our work on the production of *Aspergillus niger* mutants with enhanced xylanase activity after two-step mutagenesis using UV and the application of two different concentrations of 130 µg/ml and 200 µg/ml of N-methyl-N-nitro-N-nitrosoguanidine (NMG). A correlation was found between the morphological type of colonies surviving irradiation and xylanase production. This confirmed the appropriateness of searching for colonies with a particular morphology in future experiments, which would increase the likelihood of isolating higher active strains. A stable highly active *A. niger* mutant was obtained after induced mutagenesis with 200 µg/ml NMG at 3 h exposure. The selected mutant can be used for further analyses on xylanase biosynthesis and possible development of biotechnological processes for their production.

**Publication 3.4** presents the study of alkaline phosphatase production by *Escherichia coli* strains isolated from soil. The strains were isolated from areas of organic contamination for potential application in bioremediation of specific contaminants. All strains produced membrane-bound alkaline phosphatase and β-galactosidase. The dynamics of enzyme production was monitored in three of the isolates, which showed high baseline activity (between 10 and 20 U/ml). The synthesis of alkaline phosphatase started as early as the 4th hour and reached a maximum in the late stationary phase (24th hour in most of the strains or up to the 32nd hour) with a decrease in production thereafter. The enzymes retained relatively high activity for a long period of time, indicating relative resistance to changes in environmental conditions including pH.

**Publication 4.3** summarizes the results of the study of the lipolytic activity of 40 strains of *B. cereus* and *B. thuringiensis*. The strains of *B. cereus* produced all three types of phospholipase C. All cultures were positive for phosphatidylcholine, a specific PLC, with activity reaching 2.5 U/ml. The *B. cereus* strains also secreted sphingomyelinase C with activities between 0.36 and 0.45 U/ml. Four of the strains were selected as good producers of PLC and SMase. Additionally, phosphatidylinositol-specific phospholipase C (PI-PLC) activity was also detected in eight *B. cereus* strains. The investigated *B. thuringiensis* strains exhibited extracellular PLC and PI-PLC activities, whereas the activity of phosphatidylcholine-specific PLC was lower, 0.21 - 0.85U/ml, whereas the opposite was true for the values of PI-PLC produced.

**Publication 2.9** presents the analysis of the proteolytic activity of 166 strains of the genus *Bacillus*. This was demonstrated for 90% of the strains, distributed as follows: 110 *B. cereus* strains (66%), 32 *B. thuringiensis* strains (19%), 6 *B. sphaericus* strains (6.4%). The quantification of proteolytic activity showed high values in the range of 8-9

U/ml in: *B. cereus* No. 67(8.04 U/ml); *B. cereus* No. 88(7.74% U/ml); *B. thuringiensis* No. 4 (8.13 U/ml) and *B. thuringiensis* No. 14 (9.20 U/ml). Strain *B. thuringiensis* No. 14 was selected to optimize culture conditions (medium composition, buffering system, inoculation type) and to study the dynamics of enzyme production. Extracellular protease activity at the end of the exponential growth phase reached 15 U/ml. Partial purification of the enzyme was performed by ultrafiltration and Sephadex G-75 chromatography. The gel-filtration profile showed three peaks of proteolytic activity, suggesting the synthesis of more than one type of proteolytic enzyme by the strain. SDS-electrophoresis of the active fractions revealed the presence of two proteins with approximate molecular masses of 45 and 63 kDa, whose activity was demonstrated by zymography.

The metabolic capabilities of pseudomonads to process compounds defined as xenobiotics make them indispensable in biotransformation and bioremediation processes. Seventeen strains of *P. fluorescens*, *P. putida*, and *Pseudomonas* sp. isolated from soil were analyzed and shown to have positive phospholipase C and lipase activities in **Publication 3.9**. Phospholipase activity was highest in *P. fluorescens* strains, with maximum secretion at the 12th hour (onset of stationary growth phase). These strains were found to produce lipase with activities of 0.9 and 1.7 U/ml, with *P. fluorescens* 5B, *P. fluorescens* 1D and *Pseudomonas* sp. 1442 being the most active producers, with no correlation in the synthesis of the two lipolytic enzymes. Addition of carbon and energy sources such as xylose and rhamnose to the soybean-casein medium stimulated lipase production in strain *P. fluorescens* 5B by 29% and 17%, and addition of xylose increased production in strain *P. fluorescens* 1D by 25% as well. Lipase production in *P. fluorescens* B was entrained by 22% when xylose or arabinose was added to the medium. Phospholipase C production in *P. fluorescens* 5B was increased with the addition of xylose and arabinose to 66% and 16%, respectively, and in *P. fluorescens* 1D to 42% and 14%. In *P. fluorescens* 5B, the increase in production with the addition of xylose, arabinose, or rhamnose was 25%, 5%, and 15%, respectively. Maximum lipase secretion was recorded at the 20th hour, after that of phospholipase, which is probably related to the involvement of enzymes in providing substrates for microbial growth.

**Publication 3.12** provides data on the investigation and optimization of conditions for amylase production by *Bacillus* strains. A collection of 166 *Bacillus* spp. strains were screened for extracellular amylolytic activity. Such was found in 31% of the cultures - 61% of the *B. cereus*, 36% *B. thuringiensis*, 3% *B. sphaericus*. Activity ranged from 0.86 to 2.8 U/ml with the highest exhibited by strain *B. cereus* No 10. The enzyme began to be secreted into the culture medium in the exponential phase (8th hour/1 U/ml), but reached a maximum in the late stationary phase, 36th hour/3.14 U/ml. The secretion of the enzyme is not associated with lysis of the cells during sporulation. Enrichment of the medium with 0.1% ribose or glucose, respectively, yeast extract and Ca<sup>2+</sup> ions stimulated the amylase activity of the producer strain.

**Publication 2.13** summarizes the data from a study of phospholipase C production in one hundred and sixty-six bacterial strains belonging to the genus *Bacillus*. Initial screening on egg yolk agar showed that 87% exhibited phospholipase C activity. *Bacillus thuringiensis* strain with initial activity of 19.61U/ml was selected as the most

promising for phospholipase production. The medium composition and culture conditions were optimized to achieve higher yields of the enzyme. The highest production of phospholipase was achieved under the following liquid medium conditions: 1% yeast extract as nitrogen source; 0.5% NaCl; 0.4% glucose as carbon source; NaHCO<sub>3</sub> - 3 g/l; Na HPO<sub>24</sub> - 0.4 g/l; 1 mM ZnCl<sub>2</sub>; pH 7; inoculation with 3% (1.4 x 10<sup>9</sup> cfu/ml) subculture and 8 h culture duration. Phospholipase C production from the selected strain was scaled up in a 2l bioreactor.

**Publication 2.11** and **Publication 2.14** are related to our research on the dynamics of soil quality in urban environments under the influence of different pollutions and the search for environmentally friendly solutions for restoration and rehabilitation of terrestrial ecosystems in urban environments.

Urban habitat quality is the result of the integration of different abiotic and biotic components, such as air, soil and water quality, microclimate and vegetation. Urban soils differ from other components in the long-term retention and accumulation of contaminants. Since traffic is becoming the most serious source of pollutants in the environment, the study presented in **Publication 2.** was conducted to assess its impact on some soil properties under a pollution gradient. Soil samples were collected at distances of 7.5 m, 25 m and 50 m from two of the main boulevards in the city of Plovdiv (Bulgaria) using the transect method. The content of some heavy metals and toxic elements in soil was analyzed using ICP-MS. The data revealed that soil contamination was strongly influenced by the distance to the road, followed by wind rose and urban slope. Total number of heterotrophic microorganisms recorded during cultivation at 22°C and 37°C was used as an indicator of soil forming capacity and anthropogenic pressure respectively. A strong positive correlation was found between the two parameters. This study confirmed anthropogenic pressure (buildings and road infrastructure, deforestation) as the most important factor affecting soil quality in urban areas.

Rehabilitation and restoration of terrestrial ecosystems is a key strategy for restoring the services (goods and resources) that ecosystems provide to humanity. **Publication 2.14** presents a model study conducted in the city of Plovdiv in order to (i) assess the bioremediation capacity of some grasses and their suitability for planting lawns in urban areas (in residential and non-residential areas, along roadsides, etc.) and (ii) propose technological solutions for their practical application in urban environments. Emphasis was placed on the potential of some species of perennial ryegrass (*Lolium perenne* L.), crested wheatgrass (*Agropyron cristatum* L.), tall fescue (*Festuca arundinacea* Schreb) and bird's-foot trefoil (*Lotus corniculatus* L.). As research participants, we performed microbiological analyses of soil samples to elucidate the effect of nature-based solutions for sustainable urban soil management and quality of life improvement. A case study from the city of Plovdiv (Bulgaria) is presented, as well as an effective technological solution for creating urban lawns and roadside green buffer areas.

### **III. Materials to learning activity.**

#### **1. Co-author of the textbook "Biological membranes" :**

Denev I., Spasieva St., Stefanova D., Daskalova E., Gevezova M., **Marhova M.**, Kostadinova S. . Biological membranes. 2016, Electronic edition, Paisii Hilendarski University Press, 181 p., ISBN 978-619-202-111-5.

The textbook Biological Membranes aims to present the current knowledge of the structure and function of biological membranes based on scientific discoveries over the last few decades. The textbook includes three main sections. The first section is devoted to the development of ideas about the structure of biological membranes, their chemical composition and the structural properties of their main components. The second section is devoted to the basic functions of membranes - transport of low- and high-molecular substances across membranes, reception and transmission of intercellular and intracellular signals. The third section is devoted to specific types of membranes in the organismal world - cell membrane in archaebacteria and eubacteria, plasmalemma, internal one- and two-membrane membranes in eukaryotic cells.

The textbook is intended for students studying the discipline "Biological Membranes", but can be used by a wide range of students and professionals from all fields of biological science. It can also serve as a source of additional information for teachers of biology and natural science.

#### **2. Co-author of a textbook on Microbiology:**

C. Kostadinova, V. Gochev, **M. Marhova**, T. Girova, D. Georgiev, I. Iliev. Manual of Microbiology. **2017**. Paisii Hilendarski University Press, 265 pages, ISBN 978-619-202-240-2.

The Microbiology Manual is developed in accordance with the curricula for the training of students in Bachelor's and Master's degree programmes in 4.3 Biological Sciences, 5.11 Biotechnology and 1.3. Methodology of... at Plovdiv University "Paisii Hilendarski".

The manual includes 10 main sections - Microscopic techniques, Basic laboratory culture methods, Morphology of microorganisms, Biochemical activity of microorganisms, Identification of bacteria, Effect of environmental factors on microorganisms, Sanitary microbiology, Role of microorganisms in the cycling of substances, Medical microbiology and Microbial genetics.

The aim of the exercises is to familiarize students with modern experimental techniques in the field of microbiology and to acquire skills for analyzing and correctly reporting the results obtained.

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