

ANNOTATION OF MATERIALS

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

of Senior Assist. Prof. Dr. Ivan Zlatkov Iliev

for participation in a competition for the academic position of
„Associate Professor“, announced in State Gazette no. 92/18.11.2022 г.

Field of higher education 5. Technical sciences, professional field 5.11.
Biotechnology (Ecological and Agro-biotechnology)

The materials submitted for the competition for the academic position of Associate Professor include a monographic (habilitation) thesis, 25 scientific publications indexed in Scopus and Web of Science databases, 7 scientific publications published in non-refereed journals with scientific content, 2 technologies protected by copyright certificates for validated scientific product for implementation in practice and 2 textbooks (which were not used for the acquisition of the PhD and the academic position of Senior Assistant Professor).

The total number of citations submitted for the competition is 171, of which 134 in refereed and indexed scientific journals and 37 in non-refereed journals. h-index 6 (Scopus), with self-citations excluded.

The main part of scientific and applied research and studies can be summarized in several main topics:

1. Assessment of the ecological status of surface waters - includes analysis of the microbiome of complex reservoirs, assessment of the physicochemical parameters of the environment and quantitative and qualitative structure of phytoplankton as a biological quality element in determining ecological potential

The Earth's water resources include the usable quantities of natural water that occur as surface water or groundwater, forming aquatic ecosystems. Freshwater ecosystems contain only 0.01% of the Earth's water and occupy less than 1% of its surface. The growth and development of any country's economy is directly linked to access to clean water, but the sustainability of the ecosystems themselves depends on how they are exploited and conserved. With population growth, industrialization and the expansion of irrigated agriculture, demand for all water-related goods and services has increased dramatically, straining the capacity of freshwater basins. Knowledge of ecosystem functioning has increased considerably in recent decades, but it has not yet kept pace with the rate at which it is changing. A critical step in improving how water resources are managed is to assess their condition, and their capacity to provide the services that people will need in the years ahead.

Since its adoption in 2000, the Water Framework Directive (WFD; 2000/60/EC) has been one of the most important pieces of legislation relating to the protection, improvement and restoration of water bodies in the Member States of the European Union (EU), providing a

framework for Community action in the field of water policy. The WFD has changed the paradigm of water management from an anthropocentric perspective on water (defining it as a resource for direct exploitation by humanity) to an ecocentric perspective (where water is seen as the basis of an ecosystem). The focus has shifted to ecosystem integrity as the basis for management decisions related to water quality. The new concept of 'ecological status' was introduced for the assessment of the status of natural water bodies and 'ecological potential' for heavily modified water bodies (HMWBs). In accordance with Article 4(1) of the WFD, good ecological status (GES) was to be achieved for all HMWBs by 2015 or, at the latest, by 2027 for water bodies subject to exemptions and the principle of preventing further deterioration was to be introduced.

The presented thesis, "**Technology for Ecological Assessment and Planning of Sustainable Aquaculture in Complex and Significant Reservoirs**," presents proprietary research including appropriate indicators of ecological potential and methods for statistical analyses to determine the capacity of waterbodies for fish culture in net cages, as well as to detect trends in changing conditions at a regional scale.

Nine complex reservoirs are included in the scope of this work. They are located on the territory of the four river basin management districts in the Republic of Bulgaria, published in Annex 6 of the Regulation N-4 of 12.09.2012 for the characterization of surface waters, SG No. 79 / 23.09.2014. The main part refers to the type L11 - Large deep reservoirs, as they are the most frequently used for investment intentions for the construction of fish farms. Three of the reservoirs (Studen Kladenets, Ivaylovgrad and Zhrebchevo) are located within the scope of the East-Aegean River Basin Directorate (EARBD). The Alexander Stamboliiski reservoir is located in North-Central Bulgaria in the Danube River Basin Directorate (DRBD). Dospat reservoir is located both in the territory of the East and West River Basin Directorates. Two type L15 reservoirs (Koprinka and Ovcharitsa) in the EARBD and one type L16 dam (Aheloy) in the Black Sea Basin Directorate were also studied. The aim is to achieve maximum coverage in terms of the hydrological regime. It is directly dependent on the local climate, the flow characteristics of the inflowing water, as well as the morphology of the outflow channel and factors such as evaporation loss or direct inflow.

The activities presented are in line with the stepwise approach for the implementation of the WFD Common Strategy on the need to determine the ecological potential of complex reservoirs in Bulgaria, as listed in Annex 1 to the Water Act. Through the chosen approach, a validation of a methodology for assessing the suitability of complex reservoirs of different lake types, defined according to the current typology for the implementation of net-cage fish farming, was carried out without causing deterioration of the EP. The results obtained can be summarized in three areas:

➤ *Determination of the nutrient uptake capacity and self-purification capacity of the reservoirs through metadata analysis of the physicochemical parameters of the investigated HMWBs.* - The water retention time of each water body was determined based on its morphological characteristics and annual water inflows. It is a key parameter in determining the self-cleaning capacity and modelling the capacity to assimilate biogenic pollution, since at high rates of water exchange, the whole reservoir can be considered as a river system.

The water quality assessment of the physicochemical water quality elements (PCWQE) was conducted according to the type-specific classification systems included in Regulation No H-4/14.09.2012 on the characterisation of surface waters (Annex No 6 to Article 12, paragraph 4). Control/operational monitoring data from the National Environmental Monitoring System (NEMS) and the monitoring carried out using PCWQE indicate that in the nine reservoirs the deviation from maximum/good Ecological Potential is due to the exceedance of the limit values for single parameters according to Regulation H-4 and is not the result of an overall deterioration of the physico-chemical regime of the water bodies. Total phosphorus and its available form, P-PO₄, are the two parameters that show the most frequent deviation from maximum/good potential in the water bodies studied. Exceedance of the good EP limit is found in the reservoir. Ovcharitsa and in reservoirs of type L11 (Studen Kladenets and Ivaylovgrad) and L16 (Aheloy). The conducted studies demonstrate changes in the trophic status of the studied dams as a result of elevated TP levels, which is associated with active development and blooms of phytoplankton communities and exceeding the BEC limits for good EP.

Using PERMANOVA design, the hypothesis that the environmental conditions in a water body in the absence of external intervention remain constant over time and are directly related to its typology is proved and the effect of the choice of sampling sites is evaluated. The results are indicative of a high stability of physicochemical conditions over the study period and the absence of volley contamination, with 999 random permutations of the data series showing no significant differences by study period (pseudo-F=0.679, p(permutation)=0.639). The near-zero R values (R=0.062, p=0.001) generated when conducting the analysis of similarities (ANOSIM) confirmed the absence of differences between within-group and between-group variance. The typology applied in the country does not explain well the variation in physicochemical parameters. PERMANOVA proves that the water body, as a single object and not the type to which it belongs, has much more weight in shaping environmental factors.

➤ *Assessment of the biological self-purification capacity of reservoirs* - this is considered to be an ecologically regulated process involving two main stages related to the decomposition of organic matter by microorganisms followed by the removal of the matter from the water by uptake as biomass by producers such as phytoplankton and macrophytes. Currently, the WFD includes four biological quality elements (BQEs): phytoplankton, macrophytes, macrozoobenthos and fish, and their application is based on an intercalibrated national classification. The microbiological study carried out in the presented work focuses on the response of microbial communities to external loading and their metabolic potential in shaping the capacity of water bodies to assimilate nutrients.

Bacterial self-purification of waterbodies was evaluated in two aspects: changes in the metabolic profile and composition of bacterioplankton in response to changes in physicochemical factors and the reduction and disappearance of sanitary microorganisms such as *Escherichia coli*, coliforms and faecal streptococci. First national data on the quantitative and qualitative composition of the aquatic microbiome in large and economically important highly modified water bodies are presented. The lack of a database in water bodies with a well-studied physicochemical status limits the application of

microbiological indicators as an adjunct to water quality. Single studies have been conducted over the last 10 years, but due to the absence of a unified methodology, reported results are rarely comparable. This study complements the approach used by the conventional WFD by analysing the bacterioplankton community, incorporating key microbiological water quality indicators and analysing correlations between the microbiome in the nine dams and the physical, chemical and phytoplankton communities to assess whether the bacterial community has discriminatory characteristics with respect to water quality in the reservoir.

The present study supports the classical view of bacterial community composition (at the Division or class level), with taxonomic analysis indicating that >98% of the total sequence number is attributed to representatives of only 10 Divisions. In decreasing order, these include Proteobacteria, Actinobacteria, Cyanobacteria, Verrucomicrobia, Firmicutes, Acidobacteria, Chloroflexi, Gemmatimonadetes and Fusobacteria (Figure 14). The only exception are the 2020 samples from the reservoir. Koprinka and those from reservoir Aheloy, in which phylum Cyanobacteria was dominant, and in reservoir Ivaylovgrad, the Actinobacteria phylum displaces Proteobacteria. The differences between stations in the same water body in the two studies are due to a change in the numerical composition of individual taxa rather than to the disappearance or appearance of representatives of new phyla. The dominant complex shows sensitivity to the characteristics and changes that occur in the study reservoirs. It is characterized by well-defined spatial dynamics, also found in TVC, following the variation in FHEC.

The results obtained provide a basis for the indicators *Escherichia coli* count, faecal coliform count (FC) and faecal streptococcus count (FS) to be included in a programme of measures in the river basin management plan (2016-2021) for the East Aegean River Basin Directorate in relation to the implementation of effective and ecologically sustainable freshwater fish farming (sub- programme for conducting exploratory/own* monitoring in relation to assessing the pressure and impact of intensive fish farming in net-cage farms).

➤ *Determination of capacity of complex reservoirs for net-cage fish farming* - The term "capacity of water body" is not defined in the WFD guidance documents. The analysis conducted is an extension of a methodology to estimate the maximum phosphorus load that may enter the waters of reservoir Kardzhali during fish farming in cages, without causing deterioration of its ecological potential. The model was retested and the methodology validated for the conditions of the reservoir. The maximum fish production quotas set within the project are still applied today when renewing or issuing new permits for the use of a surface water body by the Ministry of Environment and Water.

The results presented confirm the successful validation of the model, which is a budget of the amount of phosphorus entering and leaving the reservoir so that the water body is in a "steady state". It is based on information on the hydrological characteristic determining the amounts of TP entering the water body and the morphological characteristic determining the degree of TP retention in the water column. Data from direct field measurements of TP concentrations are reliably consistent with the predicted values obtained, based on the hydromorphological characteristics of the individual SMWTs and the defined baseline EP. On the basis of the specific characteristics of the individual reservoir types and the

possibility of self-purification of their waters, the most suitable for cage aquaculture are the large deep reservoirs of type L11 (Studen Kladenets, Aleksandar Stamboliyski, Ivaylovgrad, Koprinka and Dospat), which in general have a relatively low probability of increasing eutrophication. Freshwater aquaculture in these types of reservoirs has minimal impact on the environment. The depth of the net-cages should be consistent with the depth of the reservoir at minimum water level, i.e. at the lowest water level there should be a distance of at least 3-4 m below the net-cage.

Phytoplankton and microbial communities are the first to respond to environmental changes based on multiple factors of different origins. As a primary producer, it converts inorganic substances into organic compounds during photosynthesis and transfers energy and nutrients to zooplankton and other aquatic organisms in the food chain. Temporal changes in the structure of phytoplankton are essential for metabolism in aquatic systems, with species following seasonal cycles and responding to environmental changes with changes in their qualitative and quantitative composition, so that at a given time they may reflect the physicochemical properties of the waters. This makes it a reliable biological quality element (BQE), a commonly used tool for assessing the ecological status of water bodies.

In **paper III. 1.** the identification of phytoplankton functional groups in the Kardzhali reservoir was carried out in order to improve the understanding of the seasonal dynamics of dominant species in complex and economically important reservoirs. According to our results, the dominant planktonic algal complex is composed of forty-three taxa distributed in nineteen functional groups, based on the classical Reynolds study. The most abundant representatives of functional groups F, P, K, J and the cyanobacterial group H1 are more typical of nutrient-rich eutrophic water bodies with insufficient light. The data confirm the applicability of this approach for a better understanding of environmental phytoplankton processes. This study shows that the proposed functional group model can be applied to monitoring programs, assessment of seasonal dynamics, and to collect more comprehensive information on life history strategies, habitats, and adaptations of phytoplankton communities in lakes, ponds, and reservoirs.

Studies to update the current status of phytoplankton composition were also conducted in Batak reservoir (**Article III.2.**) and reservoir Koprinka (**Article IV.1.**), included in Annex 1 of the Water Act.

Batak Reservoir is characterized by a rich algal flora, including 106 phytoplankton taxa. The class Bacillariophyceae is represented by the highest number of species (34), followed by the divisions Chlorophyta (26) and Cyanoprokaryota (17). Phytoplankton abundance (PhN) and biomass (PhB) were characterized by vertical and seasonal variations. The maximum was recorded in the autumn period in the epilimnion samples with a sharp decrease in the depth of the reservoir. Seasonally and spatially, the waters were dominated by the diatom *Tabellaria fenestrata* var. *asterionelloides* Grunow, *Fragilaria crotonensis* Kitton, the green algae *Pandorina morum* (O.F. Müller) and *Desmodesmus communis* (E. Hegewald). Cyanobacteria of the species *Aphanizomenon flosaquae* Ralfs ex Bornet & Flahault are represented with relatively high abundance. Multivariate analyses showed significant positive correlations between phytoplankton abundance and forms of nitrogen, total phosphorus, with

negative correlations between PhN and total heterotrophic microorganisms. Based on the cluster analysis, the study stations were grouped into two main clusters with similar spatial and seasonal characteristics; hierarchically, the stations in the lake zone near the dam wall and tailrace were the most distant.

In the Koprinka reservoir 109 taxa were identified, distributed in 6 divisions, including Chlorophyta (37), Ochrophyta (26), Cyanoprokaryota (22), Euglenophyta (11), Streptophyta (11) and Pyrrophyta (2). The highest biomass (PhB) and number of phytoplankton (PhN) were again found in autumn. The number of taxa identified was almost double that of previous surveys. The presence of a large number of species from the Cyanobacteria division (22 taxa) in the dominant complex, as well as the identified blooms of potentially toxic species is an indication of eutrophication. The highest species richness (46 taxa) was found at the station in the riparian zone, with phytoplankton abundance almost two to three times higher than at the other stations. Dominant species in each cluster were identified, reflecting the seasonal succession of phytoplankton. The green alga *Harriotina polychorda* dominated the summer samples at all stations. The species composition of phytoplankton, mean biomass, chlorophyll a concentration and recorded blooms of the potentially toxic species *Microcystis wesenbergii* are evidence of the eutrophic state of the reservoir over the study period.

Articles **III.3.** and **IV.2.** present the analysis of the correlations between environmental factors and their impact on the quantitative development of phytoplankton communities in the Kardzhali and Dospat reservoirs. Through correlation analysis and principal component analysis (PCA) based on the obtained correlation matrix, the main driving forces determining phytoplankton development in the two dams were identified. A total of 15 parameters were included as independent variables. The PCA results show that the variables are grouped into five principal components, which explain 82.63% and 79.91% of the total variance, for the Kardzhali and Dospat reservoirs, respectively. The data identified water transparency, electrical conductivity, pH, oxygen saturation, NH₄-N, TN, and PO₄-P as the variables with the greatest influence on the phytoplankton community in the reservoir. Significant positive correlations were found between phytoplankton biomass (PhB) with the total number of heterotrophic microorganisms.

Parameters with the greatest influence on the bacterial total viable count (TVC) include temperature, TN, and COD (**Article III.6**). The multivariate analysis (RDA) performed confirms that the location of the sampling station has a significant influence on the variables studied and that nursery aquaculture appears to be a major anthropogenic factor in the Kardzhali Reservoir. The results indicate that aquaculture-intensive reservoirs are characterized by fluctuations in physicochemical factors, biological and microbiological indicators of water quality caused by nutrient loading. No correlation was found between TP and phytoplankton parameters, which may indicate that phosphorus was not a limiting factor despite its low natural bioavailability and rapid mineralization rate, suggesting regular phosphorus input from fish feed and feces. The study confirms the reliability of PCA and other multivariate analyses for interpreting complex relationships between parameters in complex datasets in highly modified water bodies. The assessment of the ecological potential of reservoirs, according to the Water Framework Directive (WFD) criteria, is based on

common physical and chemical parameters, key for determining reference conditions for good and excellent EP in highly modified water bodies, corresponding to an ecological water status where aquatic fauna and flora live close to natural conditions, unaffected by human activities. The presence of highly invasive species such as *Dreissena spp.* may influence the relationships developed between nutrient pollution and biological indicators used in the assessment of ecological potential.

In Paper **III.14**, water column transparency (SD), total phosphorus (TP) and *chlorophyll-a* (CHL) concentrations from 49 Bulgarian freshwater reservoirs, 23 of which are invaded and 26 of which are not invaded by *Dreissena spp.* were analysed to assess the impact of invasive mussels on the potential of the three parameters to serve as indicators used to assess the trophic and ecological status of standing freshwater ecosystems. Linear regression equations for the three variables within the invaded and non-invaded water bodies were calculated and the slopes of the resulting regressions were tested for significant differences between the two groups. The presence of *Dreissena spp.* resulted in a weakening of the regression not only in the TP-CHL relationships, but also in the CHL-SD and TP-SD relationships. The group of invaded waterbodies in this study appears to represent a mixture of waterbodies with different mussel densities. Thus, the presence of mussels does not lead to complete separation, but to weaker correlations and regression equations with statistically less steep slopes; this makes the parameters still applicable (but with lower precision) for determining ecological condition.

2 Application of microbiological indicators in the assessment of environmental conditions - research focuses on the taxonomic structure of the microbiome and its physiological activity in aquatic and soil ecosystems

Microorganisms drive biogeochemical cycles in aquatic ecosystems and provide a range of ecosystem services. Bacteria are the most abundant and important group in the microbial community and are the first to respond to environmental changes by being responsible for the processes of mineralization of organic matter and its removal as biomass. Their quantitative and metabolic potential is determined by various biotic and abiotic factors, such as trophic state and temperature. However, data on the profile of microbial communities in surface water bodies in Bulgaria are very limited.

Paper III. 7. presents the first comprehensive taxonomic analysis of bacterioplankton in two large and economically important reservoirs in Bulgaria (Batak and Tsankov Kamak), which is a necessary step in understanding the microbial ecology of water bodies. The study involves massively parallel sequencing of the gene encoding 16S rRNA. The microbial communities of the two reservoirs are dominated by Proteobacteria, followed by Actinobacteria and Bacteroidetes, all of which account for over 95% of the relative abundance, despite the large hydrogeological differences of the dams. Bacterioplankton are characterized by high phylogenetic heterogeneity in taxonomic structure, being represented by a total of 211 genera. The genera *Limnohabitans* and *Rhodospirillum rubrum* have an absolute predominance in both dams. The taxonomic structure of the bacterial communities and the values of the biodiversity indices in the studied reservoirs characterize them as water bodies in an oligotrophic state.

Papers III.8 and III.25. present the first detailed analysis of bacterial diversity in two different wetlands in the Maritza River basin, taking into account the influence of the hydrological regime and rice cultivation. Wetlands are unique ecosystems combining terrestrial and aquatic habitats. As such, they exhibit some of the characteristics of each system. They are believed to be some of the most important terrestrial ecosystems, providing a huge variety of services such as pathogen removal, pollution and flood control, nutrient cycling, terrestrial runoff buffers, etc. Bacterial community structures and their differences between natural and seasonally flooded wetlands were clearly distinguished by MiSeq Illumina sequencing. The results revealed a significant difference in the bacterial community structure between the permanently flooded sediments of the “Zlato Pole” wetland and the seasonally flooded sediments of the “Tsalapitsa Rice Paddies” protected area. The multivariate analyses show that the main factors determining the bacterial community in the wetlands of the Maritza River basin are the wetland type and soil type. The dominant bacterial complex was related to ammonium nitrogen, total nitrogen and organic matter content. The number of heterotrophic microorganisms and indicators of sanitary status (FS, FC and *Escherichia coli*) in the two rice paddies near Plovdiv was higher compared to the control area Zlato Pole - the maximum was recorded in the rice paddy near the village of Tsalapitsa (C1 and C2 was 12.6×10^6 cfu.g⁻¹ and 26×10^6 cfu.g⁻¹, respectively). The bacterial complex dominated in the studied samples and exceeded the number of fungi and actinomycetes at least 1.5 times. The results confirm the importance of environmental parameters on microbial community structure, but point to the need for further and more detailed studies to reveal the spatio-temporal patterns of bacterial communities found in permanently flooded sediments and those found in seasonally drained areas.

Some of the presented scientific works are focused on the assessment of anthropogenic pressure and the possibilities for bioremediation of soils in urban conditions. The processes are hampered by the nature of urban habitats, which result from the integration of different abiotic and biotic components, such as air, soil and water quality, microclimate and vegetation. There is a clear link between the processes of urbanization and the anthropogenic transformation of the structure and functioning of landscapes, revealing an increase in the content of trace elements in the environment and a change in their load in order to restore the services provided by ecosystems. The study (**article III.9.**) reveals that the contamination of soils along the road network with Cd, Pb, Cu and Zn is directly correlated to both the distance to the road and the location of the boulevard itself according to the wind rose. This situation also affects the structure of the soil microbial community, leading not only to impairment of the physicochemical properties of the soils, but also to a reduction in their quality and functions.

Planting lawns in populated areas (in residential and non-residential areas, along roadsides, etc.) with fast-growing herbaceous species indicates good bioremediation capacity (**Paper III.10**). The focus is on the potential of some perennial grasses and their application for bioremediation of contaminated urban soils, including perennial ryegrass (*Lolium perenne* L.), crested wheat grass (*Agropyron cristatum* L.), tall fescue (*Festuca arundinacea* Schreb) and bird's-foot trefoil (*Lotus corniculatus* L.). A case study from the city of Plovdiv (Bulgaria) is presented, as well as an effective technological solution for the creation of

urban lawns and roadside green buffer areas. Vegetation cover has different effects on soil structure (porosity, aggregate stability, organic matter content, water holding capacity) and soil microorganism biocenosis, which create a more resilient soil ecosystem. There is a trend towards maintaining higher levels of biodiversity in the experimental areas compared to the background areas, indicating that these soils can store more carbon. After the reclamation activities in the newly constructed green areas, the overall activity of the communities increased at the expense of their biodiversity. Comparison of community physiological patterns by multivariate analyses showed a clear distinction between the initial and final stages of the analysis, and between the background and experimental variants. The analysis of similarity (ANOSIM) performed based on the study period, the location of the analysis points and the types of green areas supports the conclusion that the creation of new green areas (experimental plots) increases the physiological activity of bacterial communities and enhances their bioremediation capacity. The present study can be used as an approach to model microbial communities through land use change and creation of new green spaces.

Paper III.5. studies the environmental impact of greenhouse cultivation of off-season crops in terms of loss of soil quality and microbial biodiversity due to excessive fertilization. The effect that fertilization strategy has on the rhizosphere microflora after lettuce (*Lactuca sativa*) cultivation was assessed by 16S rRNA resequencing and analysis of community physiological profiles. Two lettuce cultivars were grown with bioorganic (Arkobaleno or Ekoprop NX) or conventional fertilizers. Biodiversity indices and metabolic profiles suggest that the applied agricultural practices increased the overall functional activity of the communities, but at the expense of their biodiversity. Biofertilized plots were characterized by higher physiological activity and biodiversity compared to control or conventionally fertilized soils. This is confirmed by the PERMANOVA design and the R- values obtained by similarity analysis, suggesting good ecological stability of the communities. NGS revealed that >98% of the total sequences belonged to 9 divisions including Proteobacteria, Actinobacteria, Firmicutes, Acidobacteria, Chloroflexi, Bacteroidetes, Gemmatimonadetes, Verrucomicrobia and Nitrospirae. The results show a significant reduction in biodiversity as a result of mineral fertilizer application, expressed as changes in the abundance of individual taxa rather than as species occurrence and/or replacement. Saprophytes such as *Bacillus*, *Lysoabcter*, *Pseudomonas*, *Agrobacterium*, *Streptomyces*, known to suppress plant pathogens, dominate the communities in the biofertilized soils. For this reason, the application of biofertilizers, albeit indirectly, can induce systemic resistance in cultivated plants.

A number of bacterial species are promising producers of extracellular enzymes and have significant bioremediation potential. Species of the genus *Bacillus* are among the preferred enzyme producers, mainly due to their ability to produce more than 20 g/l protein under certain conditions.

In **paper III.12.** the possibilities for the production of extracellular proteases from *Bacillus* strains are explored. A total of 166 strains of the genus *Bacillus* were analyzed, and activity was demonstrated in 90% of them. In 3% of the strains, high values in the range of 8-9 U/ml were found. *B. thuringiensis* 14 showed consistent relatively high extracellular protease activity with highest levels at the end of the exponential growth phase. Experiments were

conducted with it to optimize enzyme production by modifying culture conditions. Changing the buffer system from carbonate to phosphate and adding Mg^{2+} ions to the culture medium resulted in an increase in activity up to 15 U/ml. Partial purification of the enzyme was performed by ultrafiltration and subsequent chromatographic separation with Sephadex G-75. Analysis of the active fraction by SDS-PAGE electrophoresis indicated the presence of at least two separate proteases with molecular weights between 45 and 66 kDa. Their activity was confirmed by zymography.

Article IV.3. The extracellular activity of strains of the genus *Pseudomonas*, which are often present in highly contaminated soils, has been analyzed as they play a key role in the biodegradation of xenobiotics as the genus has the potential to degrade various carbon sources. This study focused on the lipolytic activity of non-hemolytic soil strains *P. fluorescens*, *P. putida* and *Pseudomonas spp.* All isolates produced lipases and phospholipases, with the highest relative activity found in *P. fluorescens*. Optimum enzyme production was found when the strains were cultured on soybean casein medium. Addition of an additional carbon source in the form of xylose or arabinose to the culture medium stimulated enzyme production without shifting the secretion period. It begins in the exponential phase of growth, reaching a maximum during the late exponential phase. The strains *P. fluorescens* 5B and *P. fluorescens* 1D are of interest due to the relatively high activity of the lipases produced, which may find application in water purification processes.

3. Development of technologies in the fisheries sector - the theme includes two main areas related to the farming of hydrobionts:

➤ **Technologies for fish farming**

Article III.4. The size and age at which channel catfish (*Ictalurus punctatus* Raf.) reared in the cooling pond conditions of a thermal power plant reach sexual maturity were investigated. The fish were reared in net cages. The water temperature during the winter months did not drop below 10°C, allowing the fish to feed during the autumn-winter period and generate growth. The observed weight per one-year-old catfish was 100.5 g for males and 81.2 g for females. For one-year-old catfish, body weights were 163.4 g for males and 133.2 g for females. Gonad differentiation is pronounced at one-summer-old age. GSI was 0.19 % for females and 0.016 % for males. At one-year-old age the ovaries double in size with an average weight of 0.21 g and the testes 0.03 g. The GSI has values of 0.21 % for females and 0.04 % for males. The stage of maturity of the ovaries and testes was defined as stage II. For two-year-old catfish (16-17 months of age) an average body weight of 958.9 g for males and 894.8 g for females was found. GSI was 1.68% for females and 0.15% for males. The ovaries were at III-IV stage of maturity and their majority consisted of early vitellogenic follicles. At 18-19 months of age, the ovary was at stage IV maturity and vitellogenic follicles were the major fraction in the ovary. Channel catfish reached sexual maturity at age two at an average weight of 1,007 g for males and 985 g for females, with readiness to spawn in late spring (May-June) by the beginning of the third growing season.

Article III.15. The influence of the stocking material composition and its density on primary productivity in earthen-type ponds is investigated. The stocking options included different stocking ratios of carp (*Cyprinus carpio*) and bighead carp (*Aristichthys nobilis*) ranging

from 3:1 to 1:3. One- and two-year-old specimens of grass carp (*Ctenopharyngodon idella*) and bighead carp (*Aristichthys nobilis*) were added to each of the variants at similar abundance. Survival and yield analyses indicate that the 3:1 stocking density of carp and bighead carp indicates the best survival of the bighead carp. This density ratio between the two species is more suitable to generate mutual benefits. Despite regular artificial feeding, the larval yield of carp and grass carp correlated significantly with net primary plankton production, while the yield of bighead carp showed no correlation due to detrital feeding.

The European catfish (*Silurus glanis* L.) is one of the most commonly farmed predatory fish in warm-water ponds. It is used as a bio-meliorator in ponds intended for breeding various fish species with the main objective of regulating the amount of weed species. In aquaculture, it is grown in monoculture or polyculture. Its food spectrum includes a wide range of aquatic organisms such as crustaceans, mussels, fish, etc. When reared in polyculture, the number of catfish is determined either by the amount of weed fish in them or by how efficiently they can utilise them, turning them into valuable meat. In this respect, its preference for consumption of one prey or another is of considerable interest (**Paper III.16**) - a problem that has important practical implications. The aim of this study was to determine the selectivity index of the European catfish (*Silurus glanis*) when reared in polyculture with common carp (*Cyprinus carpio*) and weed fish (*Pseudorasbora parva*). Positive selectivity index values were found for *P. parva* (+0.2575) and negative values for *C. carpio* (-0.1163). The data showed that under controlled laboratory conditions, the yearling European catfish showed a greater affinity for *P. parva* than for carp, given that 60.25% of the prey consumed was weed fish and the remaining 39.75% was carp.

Paper III.23. analyses the growth rate and food conversion ratio of one-summer-old European catfish (*Silurus glanis*) when fed naturally with common carp (*Cyprinus carpio*). When European catfish were reared in polyculture with yearling carp (*Cyprinus carpio*), results indicated that a growth rate of 25% was achieved within a thirty-day period in a predator-prey biomass ratio of 1:3. And its growth rate for the first variant was 0.52 cm or 2.25%. The feeding coefficient for European catfish when feeding on carp is in the range 4.3 - 4.7.

Paper III.19. The variation in body weight of cultured hydrobionts depends on many external factors: density, quantity and quality of food and hydrochemical characteristics are the most important, bearing in mind that individual potential is also important for the growth rate of each specimen. The body length (SL, cm) and growth rate (BW, g) characteristics of European catfish (*Silurus glanis*) one-summer-old broodstock reared in earth-type ponds show considerable variation. Fish with a body weight below 50 g accounted for 88.6 % and those with a body weight above 50 g for 11.4 %. A significant proportion of the yearling fish (50%) weighed less than 10 g, reducing their chances of reaching a consumptive growth rate in the following year. The correlation between weight gain and body length can be described using an equation of the type $BW=0.0109SL_{2.8581}$ ($r=0.9862$) in the under 50 g body weight group and $BW=0.0142SL_{2.7809}$ ($r=0.951$) in the over 50 g body weight group. The Fulton's coefficient was determined for the two groups of yearling fish studied. According to this index, there is a difference between the two analyzed groups which is statistically significant at $p<0.001$. The characteristics of body length and body weight standard of yearling catfish

produced by one parent and reared under identical conditions differed significantly in terms of body weight (BW, g) and body length standard (SL, cm), 154.33% and 41.49%, respectively. Fish with body weight less than 50 g represented 88.6% and those with body weight more than 50 g represented 11.4%. It can be assumed that the reported variations in body weight and body length of yearling European catfishes are a consequence of both the different growth potential of each individual within its species, which is a factor of great importance and is typical for predatory fish, and the specific rearing conditions.

In aquaculture, very often fish have to undergo various manipulations related to artificial propagation, surgical operations, tagging, measuring, transporting, blood sampling, etc., which can lead to stress, traumatization or even death. All manipulations are difficult due to the energetic resistance of the fish, which can result in injuries, surface and internal bleeding, incomplete hatching of eggs and other negative consequences. To a large extent, these negative effects can be minimized by the use of various types of anesthetics. Their application immobilizes the fish, reduces stress and ensures humane treatment of the fish.

The aim of the research in **Article III.22.** and **Article IV.7.** is to investigate the use of clove oil, a natural product that has long been used in medicine, cosmetics and the food industry, as an anesthetic in predatory fish species, including pike (*Esox Lucius* L.) and channel catfish (*Ictalurus punctatus* Raf.). Experiments conducted under controlled laboratory conditions confirm the good potential of using clove oil as an anaesthetic in performing various manipulations in both species. The effect of applying the solution in concentrations of 20 $\mu\text{l.l}^{-1}$ to 80 $\mu\text{l.l}^{-1}$ was tested. During the course of work, the behavior of the fish was monitored and the time taken to reach complete anaesthesia and recovery time was recorded. Low concentrations (20 $\mu\text{l.l}^{-1}$) did not achieve complete anaesthesia in both species studied and had only a sedative effect. The best results in terms of time to reach phase 4 (full anaesthesia), where fish lie on the bottom and do not respond to handling, and time to full recovery were observed at concentration (60 $\mu\text{l.l}^{-1}$). At this concentration, pike reached phase 4 of anaesthesia, complete loss of reactivity, in 7.00 min, and recovered in 4.43 min. Channel catfish became anaesthetized for an average of 3 min and recovered in 3.24 min. There was an inverse relationship between recovery time and clove oil concentration.

➤ **Survey of freshwater crayfish populations in Bulgaria and assessment of their economic potential**

In Bulgaria, the populations and distribution of crayfish are very poorly studied. Existing data are limited and, in many cases, considerably outdated, or are primarily related to the study of epibionts on them. Two of the species of crayfish (noble crayfish, *Astacus astacus* (Linnaeus, 1758) and stone crayfish (*Austropotamobius torrentium* (Schrank, 1803)) inhabiting the country's water bodies are characterized by severely reduced abundance, which is why various bans and restrictions on their capture exist in many countries. They are included in Annex III of the International Convention on the Conservation of European Wild Flora and Fauna and Natural Habitats, ratified by Bulgaria (State Gazette No. 23 of 10.03.1995). In Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora, the river crab is listed in Annex 5 and the stone crab in Annex 2. The IUCN Red List classifies the river crab as Vulnerable (VU). In Bulgarian legislation it is listed in the

Biodiversity Act in Annex 4 as a species under conservation and regulated use by nature. The presented publications (III.17.; III.20.; III.21.; III.24.) deal with new localities of noble crayfish (*Astacus astacus*) and stone crayfish (*Austropotamobius torrentium*). The surveys established some general characteristics of the populations, such as relative weight and size of the individuals. Their relative abundance (CPUE), males to females ratio, and the behavior of the species when competing for shelters, was also determined

Paper III.18. presents data on a new locality of the narrow-clawed crayfish (*Astacus leptodactylus* Eschscholtz, 1823) in a quarry pond located near the village of Boshulya. The number of crayfish caught (925) indicates a high population density. Individuals with a body weight between 20.1 and 30.0 g. predominate. They represent 38 % of the total catch. The groups with the lowest percentage (1%) are those with a body weight of 70.1 - 80.0 g and those weighing more than 80 g. The absolute fecundity of the population was determined based on a sample of 24 sexually mature females. The mean number of ova was 228 with a range of 144 - 294. The gonadosomatic index (GSI, %) for the sample studied was 3.92%. The observed relative abundance of the lake crayfish population in the quarry pond of village of Boshulya is high (mean CPUE 14.69), indicating that it has significant economic potential and can be exploited for industrial fishery.

4 Contributions in the field of microbial pathogenesis

Paper III.11. Concentrated extraction products (concrete, resinoid, extract with 1,1,1,2-tetrafluoroethane) from the leaves of three Bulgarian types of tobacco (*Nicotiana tabacum* L.) – flue-cured Virginia, Burley and Oriental, have been obtained. The products, as well as the respective essential oils, have been characterized by their chemical composition (mono-, sesqui- and diterpenoids, phenylpropanoids, nitrogenous compounds, etc., and phenolic acids) and activities (antimicrobial, anticorrosion). The studied extracts demonstrated antimicrobial activity against Gram-positive, Gram-negative bacteria and yeasts. The obtained results reveal the differences both on product and raw material (tobacco type) basis, and mark the possible fields of their application.

Paper IV.4. The present study focused on antibiotic and serum resistance of strains of the family. Enterobacteriaceae associated with urogenital tract infections in the community. *Escherichia coli* was the most commonly isolated etiological agent (64.8%), followed by *Klebsiella spp.* (17%) and *Proteus mirabilis* (10.37%). Antibiotic susceptibility tests showed high resistance to ampicillin (49%), micilinam (71%), doxycycline (41%) and high susceptibility to cephalosporins (cefuroxime 84.6%; cefoxitin 83.7%; cefotaxime 91.5%; cefepime 87.7%) and fluoroquinolones (ciprofloxacin 85%, norfloxacin 79%, levofloxacin 83%;). Significant resistance was found to nitrofurantoin (24%). Among the strains tested, 8.5% produced extended-spectrum beta-lactamases (ESBLs). Serum susceptibility testing of the strains showed that 84% of the strains were resistant to the bactericidal activity of normal human serum. Evidence suggests that resistance to the Complement is most likely one of the obligate virulence factors for most *Enterobacteriaceae* strains associated with urogenital infections. The susceptibility profile of the *Enterobacteriaceae* strains studied confirms the need for continuous updating of antibiotic resistance data and virulence patterns of etiological agents of urogenital infections.

Article IV.5. The representatives of genus *Enterococcus* are increasingly associated with infections of the urogenital tract. *Enterococcus faecalis* and *Enterococcus faecium* species are the third most commonly isolated pathogens in catheter-associated urinary tract infections (CAUTI). Both species are capable of forming biofilms, with *E. faecalis* is characterized by a higher frequency of isolation. This study investigated the antimicrobial resistance and biofilm-forming ability of 72 *Enterococcus faecalis* strains collected over one year from the urogenital tract of outpatients. The results showed that urinary tract infections (UTIs) caused by enterococci were more common among children up to 10 years of age, whereas genital tract infections (GTIs) were most common in women of reproductive age. Overall resistance is low, with higher levels in strains associated with the urinary tract compared with the genital tract. Results showed 100% susceptibility to penicillins, which are the most effective agents for the treatment of infections caused by *Enterococcus faecalis*. Resistance to fluoroquinolones was less than 19%, with marked cross-resistance. Biofilm formation was detected for 26 % of the strains tested after 24 h cultivation on soy-casein medium, with OD₆₃₀ values for biofilms in the range 0,050- 0,200. This categorizes the strains as low biofilm forming strains. The susceptibility profile of the strains tested in this study and their ability to form a stable biofilm confirm the continued need for mandatory determination of antibiotic resistance prior to prescribing of drugs by physicians.

Article IV.6. Vaginal yeast infections are one of the most widespread infection diseases among women. The present study is focused on the taxonomic composition, antifungal resistance, and some virulence factors of *Candida* strains isolated from samples of outpatients. During one year period from April 2016 to March 2017 the 97 *Candida* spp. strains were collected from vaginal, cervical, and urethral secrets of outpatients at IMDL “Chronolab” Plovdiv. The majority of isolates were identified as *C. albicans* (84%), followed by *Candida glabrata* (7%), *Candida krusei* (4%), *Candida parapsilosis* (3%), and *Candida tropicalis* (2%) and the most affected age group is women between 21 and 40 years old. Antifungal resistance was low and mainly associated with *C. glabrata*, with total susceptibility to the tested antifungal drugs over 95%. Analysis of the hydrolytic enzyme activities and the biofilm formation abilities showed that only 8% of the strains produced gelatinase and phospholipase, 6% produced caseinase, and 5% esterase. Seven of the tested *Candida* strains (7.2%) formed stable biofilm after 24 h cultivation in Sabouraud dextrose broth supplemented with 6% glucose. This study revealed no significant correlation between the antifungal susceptibility and the studied virulence factors of *Candida* spp. isolates from the genital tract of outpatients.

5. Technologies implemented in fish farming practice

5.1. A technology for rearing pike (*Esox lucius* L.) in fish farming ponds is developed. It includes: a brief characterization of the species, its environmental requirements and the importance of the habitat for rearing. The technologies for natural, semi-artificial and artificial propagation and rearing of the species are systematized. Stocking schemes, its role as an additional species in the stocking and expected yields at different age groups, and the importance of the species as a natural regulator of weed fish in ponds are discussed.

5.2. A System of rules for Good Aquaculture Production Practices (GAPP) is developed describing the recommended practices for common carp farming at the farm level, in a sustainable aquaculture setting and in compliance with environmental requirements. The document develops the basic requirements for quality control of the different production activities in accordance with the principles of good production practice. Methods of propagation and rearing of carp, feed, nutrition, prophylaxis and application of drugs and transport of stocking material and finished products are detailed. The organization and management of the carp farm are presented.

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Plovdiv

Prepared by:

Dr. Ivan Zlatkov Iliev