

DAIRY PLANT RAW SEWAGE LONGITUDINAL INFLUENCE ON RIVERINE WATER QUALITY, BOTTOM INVERTEBRATES AND THE MASS DEVELOPMENT OF HEALTH-THREATENING MICROORGANISMS IN AGRICULTURAL RIVER REACH, NE BULGARIA

Dimitar DOYCHEV^{1*}, Rositsa DAVIDOVA¹ & Lidiya TANEVA¹

¹Faculty of Natural Sciences, Konstantin Preslavsky University of Shumen, Address: 115 Universitetska Str., 9700 Shumen, Bulgaria; d.doichev@shu.bg, r.davidova@shu.bg, l.dimitrova@shu.bg

*Corresponding author: d.doichev@shu.bg

Abstract: Dairy plants (DPs) are the most significant polluting source of wastewater in Europe causing overall degradation of freshwater ecosystems dictated by the quality characteristics of the effluent which is more polluting than municipal sewage waters. This leads to environmental problems related to various biological quality elements such as macroinvertebrates and health hazards by facilitating the increase of coliforms. The paper aims to assess the longitudinal macroinvertebrate and water quality alteration in a downstream direction from a DP and to point out the most abundant genus of microorganisms identifying potentially hazardous species in agricultural areas from which nutrients are leaching. To achieve this a river reach with 3.3 km length was investigated for 3 years. Four sampling sites were established for invertebrate and water samplings. The sites were monitored in the spring-summer and autumn periods. The microbiological survey was conducted once from the most polluted sites and remote sensing data was used to evaluate the seasonal dynamics of nutrient leaching upstream from the dairy plant. Multiple factor analysis (MFA) was used to determine the most important environmental parameters for the biological indices and the most influenced one from normalized difference in vegetation cover and moisture content, in the surrounding arable lands. Our results demonstrated that ammonia was the only parameter longitudinally decreasing downstream from the DP and was the nutrient with the strongest influence on bottom invertebrates along with phosphates. Conductivity, phosphates, nitrites, and sulfates were longitudinally increasing partly because of microbiological and macroinvertebrate activity. Health-threatening microorganisms were found in great quantities for 1.5 km downstream of the DP and macroinvertebrates did not recover for the 3.3 km long river stretch. The approach of this study could be beneficial for the planning of monitoring a heavily polluted water bodies from DPs in terms of defining the right distance for quality assessment.

Keywords: dairy plants, rivers, macroinvertebrates, microorganisms, *Escherichia coli*, nutrients, NDVI, NDMI

1. INTRODUCTION

A variety of anthropogenic activities lead to the acceleration of environmental degradation (Mazari et al., 2024). Dairy plants (DPs) are an example considering surface water impacts. They are the most significant source of wastewater in Europe because in parallel to industrialization, the production of milk and milk products increased by 2.8% per year. In 50% of the cases, the waste is discharged without any treatment (Slavov, 2017), and adding to the significant increase in the number of DPs worldwide, the consequence is the negative effect on biodiversity

since 4 to 11 million tons of sediments and wastewater are generated annually (Ahmad et al., 2019). This leads to the overall degradation of freshwater ecosystems, dictated by the quality characteristics of the effluent. DP discharge is specific with the elevated temperature, high concentrations of organic compounds, nutrients, pH varying in a wide range, high biological oxygen demand (BOD), chemical oxygen demand (COD), as well as increased amounts of total nitrogen, total phosphorus, fats, oils, carbohydrates, and proteins. These variables are dependent on the operating system of the respective facility and the types of dairy products it produces (Porwal et al., 2015; Slavov, 2017).

Wastewaters are classified into 3 main groups: production waters (for cooling), cleaning waters (solid organic particles and production residues), and sanitizing waters (may contain soaps and other cleaning agents). The most heavily contaminated component mixing with the "cleaning waters" is the whey. It forms between 85% and 95% of the volume of all wastewater and 55% of milk components (Slavov, 2017). Whey is 60 to 80 times more polluting than municipal sewage waters (Raghunath et al., 2016). It has elevated COD and BOD, which in 90% of the cases is a consequence of the available lactose. If COD is considered the dependency is on 12% of the available concentration of proteins (Slavov, 2017).

Other physicochemical parameters regularly measured during water monitoring programs, such as the amount of nitrogen and phosphorus differ for each type of runoff generated by DP operation. Nitrogen occurs mainly in the form of amino groups in proteins but can also be found in the form of ammonium ions, nitrates, and nitrites. As for phosphorus, it is mostly inorganic and detectable in the form of phosphates and diphosphates (Demirel et al., 2005).

The Black Sea basin management district where the studied river reach is located has the lowest percentage (30-40%) of rivers achieving good ecological status (GES), according to the Directive 2000/60/EC of the European Parliament and of the Council of October 23, 2000 (WFD). This is the worst result of the four river basin management districts in Bulgaria, with an average result for the country of almost 50% (EEA, 2018). That percentage is slightly higher than the overall European results from 2015 when 40% of the water bodies achieved GES because European rivers are affected by multiple anthropogenic pressures such as water scarcity, hydro-morphological alteration, invasive plant and animal species, diffuse pollution, and point source pollution. However, 40% of the EU land cover is occupied for agricultural purposes responsible for the diffusive enrichment of water bodies with nutrients, pesticides, river siltation, and other hydro-morphological alterations (Schürings et al., 2024) partly caused by soil erosion (Gümüş, 2023). Nevertheless, a point-source pressure related to the dairy industry could be highly polluting and therefore determinant for the environmental status of surface waters since hard-to-survive conditions are created for aquatic organisms.

Ecological assessments in the EU countries rely precisely on aquatic organisms or the so-called biological quality elements (BQEs) which are heavily influenced by the toxicity of DP discharge quality (Ahmad et al., 2019). An example of responsiveness to pollution BQE is macroinvertebrates (Kucuk & Albaz, 2008). They reflect the change in nutrient conditions (Moskova et al., 2008) even to the scale of

minor changes in the ammonia concentrations that could cause difficulty in detecting chronic toxicity or physiological stress (Doychev & Taneva, 2025).

In addition, the contamination of lotic ecosystems from DP effluent causes the development of bacterial colonies known as sewage fungus which can be dominated by *Sphaerotilus natans* (Shete & Shinkar, 2013) and overall intensive growth of bacteria (Ahmad et al., 2019). When wastewater is untreated the consequence could include a health risk (Tikariha & Sahu, 2014) since microorganisms such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus hirae*, *Enterococcus faecium* etc. could be found (Shivsharan et al., 2013).

Bearing in mind all the mentioned, the present research aims to evaluate the spatiotemporal longitudinal influence on macroinvertebrates, downstream of a DP discharging untreated wastewaters, based on analysis of the environmental variables fluctuations in a river reach with a length of about 3.3 km during spring-summer and autumn periods. In addition to the main goal, we pursue an understanding of the seasonal differences in nutrient content within the water column, upstream of the DP, by using remote sensing instruments to extract data for the agricultural surroundings and to indicate by classical microbiological methods the presence or absence of pathogenic microorganisms that could create health risk.

2. MATERIALS AND METHODS

2.1. Study area

According to the River Basin Management Plan (RBMP) (2016), Kriva Reka River is from the type R11 "small and medium-sized Black Sea rivers". This type is specific with a slight slope and a slow current. The predominance of fine substrates such as sand, silt, and clay, as well as the lack of heterogeneous morphology, is also characteristic. The type is indicated to be distributed below 70 m above sea level (a.s.l.), although this northernmost tributary of the Provadiyska Reka River is at an elevation of over 200 m a.s.l. The specific sunken trapezoidal bed for the river type is also characteristic of the Kriva Reka River.

As for the typical concentrations of biogenic elements in the riverbed, total phosphorus is indicated as a content between 0.02 and 0.09 mg/L, and for phosphates (PO_4) between 0.06 and 0.51 mg/L. Nitrate nitrogen, nitrite nitrogen, and ammonium nitrogen are respectively in the range of 0.5 - 3.2 mg/L, 0.01 - 0.04 mg/L, and 0.06 - 0.16 mg/L; when there is no diffuse or point source pollution. Electrical conductivity (EC) also varies widely and can reach values more than 900 $\mu\text{S}/\text{cm}^2$. The pH is slightly alkaline between 7.88 and

8.23, and dissolved oxygen (DO) ranges from 6.2 to 9.4 mg/L under normal conditions (RBMP, 2016).

The Kriva Reka River enters 3 WFD water bodies (BG2PR600R014, BG2PR600R013, BG2PR600R012) with a total length from source to mouth of 43 km. The studied water body (BG2PR600R013) is natural and located in the middle of the river course with a 19 km length.

The sampling site located in the uppermost part of the river is Kriva Reka River 0 (K0) at an altitude of 240 m (Figure 1), immediately before the Kriva Reka village. The predominant purpose of the surroundings of the river is for agriculture, mainly intensive and monocultural. The morphology of the riverbed has the following distribution of substrates: microlithal (2-6 cm) - 10%; akal (0.2 - 2 cm) - 10%; agrilal (silt) - 80%. Fallen trunks and branches covered 50% of the water surface, while macrophytes 20%.

Sampling site Kriva Reka River 1 (K1) is located at the same altitude, immediately after the point of discharge of the DP in the Kriva Reka River, at about 20 m downstream from K0 (Figure 1). The bottom substrate is completely covered by sewage fungus. Fallen trees and branches cover approximately 20% of the water column. The oiliness of the substrate is observed, and a strong smell is released. Bubbles of gas are released from the bottom substrate by the accumulated sediment, which in places reaches about 0.6 m.

Sampling site Kriva Reka River 2 (K2) is located at 1751 m downstream from K0 at 226 m a.s.l

(Figure 1). The bottom substrate is completely covered by sewage fungus. Fallen trees and branches covered about 20% of the water current. The oiliness of the substrate is observed, and a strong odor is released when disturbing the sediment. The site is almost completely shaded. Concrete threshold and large stones in the riverbed, under the sewage fungus, provide good aeration of the passing water masses and heterogeneous hydrological conditions, which are not typical for other sites.

Sampling site Kriva Reka River 3 (K3) is located 3300 m downstream from K0, at 215 m a.s.l (Figure 1). Microlithal (2-6 cm) dominates the site, constituting about 40% of the bottom substrate. Mesolithal (6-20 cm), akal (0.2-2 cm), psamopellal, agrilal and xylal (fallen trees and branches) are also available, all with 10% representation, except for the silt (agrilal) which reached 15%. The psammal (sand) was the scarcest - 5%.

2.2. Microbiological analysis

The microbiological analysis based on one mixed sample of sewage fungus was indicative. The sample is mixed from K1 and K2 substrates. The material was taken in November 2022 and analyzed in laboratory conditions.

The development of microorganisms took place in several nutrient media varieties: Pseudomonas agar base, Azide maltose agar, MacConkey agar,



Esri, TomTom, Garmin, FAO, NOAA, USGS; Esri, Intermap, NASA, NGA, USGS; Esri, TomTom, Garmin, Foursquare, GeoTechnologies, Inc.

Figure 1. Map of the studied sampling sites in Kriva Reka River. The green polygon represents the area from which the satellite data were retrieved.

Chromogenic coliform agar, and MRS medium. The method consisted of several successive stages that included dilutions, inoculation on solid nutrient media, and incubation of the seeded dishes followed by grown colonies counting.

Faecal coliforms were enumerated from the 5th dilution with the most probable number. *Escherichia spp.* was enumerated on MacConkey agar (Merck, Darmstadt, Germany) and Chromogenic coliform agar. *Pseudomonas spp.* is listed on Cefrimide Agar (Merck KGaA, 64271 Darmstadt, Germany). *Enterococcus spp.* in a selective medium Azide maltose agar. MRS medium (de Mann Rogosa Sharpe, Biolife 272-20128, Milan, Italy) was used for lactic acid bacteria isolation.

Macromorphological characterization of the grown colonies was done using an OPTIKA stereomicroscope and a digital microscope camera Dino Capture 2.0 Version 1.3.8. The species-level identification of the isolated strains was performed solely for *Escherichia spp.* using identification technology with a Manual Microbial Identification System MicroLog M® BIO45101 Biolog Inc and GEN III software. The isolated strains were screened on BL4021502 Tryptic Soy Agar, cultured for 48 hours at 37 °C, and then subjected to Gen III plaque identification to identify Gram-positive and negative aerobic bacteria.

2.3. Macroinvertebrate sampling and determination

In total 8 biological samples with macroinvertebrates were collected from K0 and K3. They were taken during the three years of the study from June 2022, November 2022, June 2023, and May 2024. The number of samples taken is relatively low, since at K1 and K2, macroinvertebrate representatives are not present, but only sewage fungus is found, which covers the entire bottom substrate. Only in June 2023 at K1 and K2 *Chironomus sp.* were registered in small numbers which did not require taking samples but only determining their numbers *in situ*. A standard hydrobiological “kicking” net with a mesh size of 500 µm and a frame size of 25 × 25 cm was used. A single net dip and kick covers an area of 0.125 m² and corresponds to one sub-sample. Ten sub-samples were taken in one biological sampling (1.25 m²) from each 30 m long site. The specific microhabitats were sampled proportionally to their distribution at the sampling site. In addition, when necessary, individual elements of the substrate are washed to obtain a more complete taxonomic picture (Cheshmedjiev et al., 2011).

Subsequently, probes were washed and cleaned on the field from mechanical impurities. The general taxonomic level was family, except for Tricladida and Oligochaeta identified as such. Some taxa (*Sphaerium*

sp., *Chironomus sp.*, *Nepa cinerea*, and *Ranatra linearis*) were identified to genus and species level with a ×20-×80 magnification stereomicroscope (BRESSER, Researcher ICD LED).

2.4. Physicochemical parameters

In situ measurements include water temperature (T), pH, DO, and EC. All field measurements were conducted with a multiprobe Senso Direct 150 Lovibond (Tintometer GmbH, Dortmund, Germany) at all sites when water samples were collected.

In total 19 water samples for standard nutrient analyses were taken from all sampling sites, according to Bulgarian State Standard EN ISO 5667-6:2016 in April 2022 (excluding K3), June 2022, November 2022, June 2023, and May 2024. Phosphates (PO₄), nitrites (NO₂), nitrates (NO₃), ammonia (NH₃), and sulfates (SO₄) were measured in the laboratory. Those variables were analyzed using standard colorimetric analysis referring to the guidance for photometer Hanna HI 83200 (Hanna Instruments, Rhode Island, USA).

2.5. Ecological status assessment

The taxonomic composition of bottom invertebrate fauna specified by genus and family levels allows the use of biological indices for the so-called ecological or environmental status (ES) assessment. According to Ordinance H-4/2012, the ES is a result subsequently calculated from indices using BQEs, in our case macroinvertebrates (Government of Bulgaria, 2012). The ordinance follows the European legislation and in particular the WFD and classifies surface waters in Bulgaria, according to their BQEs results in high or better than good ecological status, good ecological status (GES), moderate ecological status, poor ecological status, and bad ecological status.

Physical and chemical parameters or physicochemical quality elements (PCQEs) are classified as accompanying information that can serve as supporting data for the ES assessment. Their results are classified into 3 groups of values: high, good, and moderate ES (European Commission, 2005; Government of Bulgaria, 2012).

The following indices were calculated from bottom invertebrate fauna results:

- Total number of taxa (S) (Cheshmedjiev & Varadinova, 2013) for which families with a single individual were counted, following Guareschi et al. (2017).
- Ephemeroptera, Plecoptera and Trichoptera richness (EPT) is an index which was used since it is stable in unpolluted sites (Cheshmedjiev & Varadinova, 2013) and sensitive to nutrient content in the

neighboring Kamchia River basin (Moskova et al., 2008).

- Relative abundance of EPT (%EPT).
- Adapted biotic index (BI) has been preferred and used in Bulgaria for more than 24 years in surface water state monitoring programs (Doychev, 2023).
- Biological Monitoring Working Party (BMWP) is an index developed for UK streams (Hawkes, 1998). The interpretation is by Kucuk and Alpbaz (2008).
- Average score per taxon (ASPT) is a derivative of the BMWP. It is less influenced by the number of taxa. The interpretation of the scores is under Hieu et al. (2016).
- Dominance index (DOMN) (Simpson, 1949).

2.6. Satellite data

Normalized difference vegetation index (NDVI) and normalized difference moisture index (NDMI) were calculated for the agricultural surroundings 1 km before K0 (Figure 1) to explain nutrient content regimes with diffuse origin in the water body before Kriva Reka village. For this purpose, we used the Sentinel-2 LMA dataset. Mean values for the pointed surface area (Figure 1) of NDVI and NDMI were gathered from satellite observations when cloud coverage was lower than 10% for 1 month before every water sampling event. Afterward, a mean score was defined for the 5 samplings at K0 and used in the data analysis.

NDVI was used since it is an index quantifying vegetation cover. The index normalizes green vegetation reflectance in near-infra-red (NIR) wavelengths (around 0.86 μm) with chlorophyll absorption in red wavelengths (0.66 μm). NDVI has been widely applied for about 50 years for a variety of purposes such as estimation of crop yields, dry mass residue (Gao, 1996) and for water quality assessments in rivers (Muthukrishnan et al., 2007) and dams (Chu et al., 2013).

NDMI uses NIR and slow-wave infrared (SWIR) for the plant moisture content calculation. The central wavelength of SWIR measures the reflectance from the vegetation and the mesophyll at about 1.61 μm while NIR is influenced only by the dry matter and the structure of the leaves. This method was developed by Gao (1996). NDMI was applied in the study to test how the moisture in the vegetation of the agricultural surroundings can correlate with nutrients in the slowly flowing river. For the rest of the sampling sites NDVI and NDMI were not used because of the DP heavy pollution.

2.7. Data analysis

The data analysis of water quality parameters, satellite data, and macroinvertebrate indices was conducted using multiple-factor analysis (MFA). The

use of regression modelling seemed inappropriate due to the limited samplings. Microbiological results do not allow comparisons with other variables and were not used for MFA.

MFA was chosen due to its great potency in analyzing data differing in nature, considering the simultaneous influence of every group of variables. All types of parameters are active and are normalized separately due to the possibility of varying weighting values between different groups and therefore ensure harmonized influence from each data set (Kassambara, 2017).

In our research, MFA was applied to extract the general information from quantitative variables in two datasets. The main one includes physicochemical parameters and biological indices from all sampling sites and the other one contains satellite data and physicochemical parameters solely for K0. The analysis was conducted in R environment (R version 4.2.3, R Core Team, 2023), using “factoextra” and “FactoMineR” packages. The significance ($P < 0.05$) of the variables for principal components 1 (Dim 1) and 2 (Dim 2) were determined using the “dimdesc” function in “FactoMineR” (Kassambara, 2017).

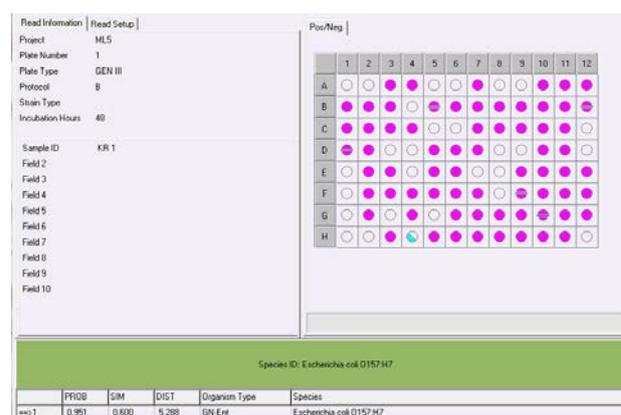


Figure 2. Manual Microbial Identification System results demonstrating the species ID and the probability (PROB – 0.951 = 95%) of the result.

3. RESULTS AND DISCUSSION

3.1. Microbiological contamination as a health threat

Microorganisms isolated from the sewage fungus from K1 and K2 are represented in colony-forming units (cfu) per cm^3 . The most numerous were *Escherichia coli* O157:H7 (Figure 2) with 12.1×10^5 cfu/ cm^3 isolated on Chromogenic coliform agar, followed by *Enterococcus* spp. with 10×10^5 cfu/ cm^3 . *Escherichia* spp. was registered on MacConkey agar as well, but in less quantity - 5.2×10^5 cfu/ cm^3 . *Pseudomonas* spp. also was in large quantities reaching 8.7×10^5 cfu/ cm^3 . MRS

medium was the only selective nutrient medium that did not register the growth of microorganisms, particularly lactic acid bacteria.

Escherichia coli O157:H7 causes diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS). The entero-hemorrhagic bacterial strain acts by infecting the digestive tract and causing abdominal cramping with hemorrhagic diarrhea. In addition, *E. coli* O157:H7 induces enterohemorrhagic disease, which can cause systemic disease through HUS, which manifests as hemolytic anemia, thrombocytopenia, and acute renal failure. HUS can result in both acute, potentially life-threatening illnesses and chronic illnesses throughout life (Atnafie et al., 2017).

The discovered bacterial strain, although not registered in potable sources but in recreational ones, poses a risk to human health by itself (Jamieson et al., 2003) and because it generates two cellular toxins (Tarr, 1994).

A clear limitation of our study is the lack of information about microorganism species diversity at every site from every sampling event. This will enable more detailed and profound statistical analysis and assure a better understanding of water quality parameters dynamics etc.

3.2. Macroinvertebrate responses

The results of the macrozoobenthic survey recorded a total of 26 taxonomic groups before the point of discharge of the DP. At K0 the most numerous taxa were Gammaridae and Oligochaeta. The latter dominated the substrate in November 2022 and June 2023, while amphipods dominated in June 2022 and May 2024. The Orders Ephemeroptera, Trichoptera, Megaloptera, and Coleoptera were found only here (Table 1).

Ten individuals from order Diptera (*Chironomus* sp.), were registered once at K1 during the survey in June 2023. These individuals were probably brought by the current and did not establish a stable population, bearing in mind that invertebrates were not found during the rest of the samplings. At K2 *Chironomus* sp. was also once found during the same sampling event, but there the genus was more abundant (Table 1).

At K3, some taxonomic recovery of invertebrates was observed, and 16 families were recorded for the entire study period and all 4 samplings. Dipterans from the Chironomidae family dominated the samples from June 2022 and 2023. Gammaridae were most numerous in the autumn of 2022 and consequently became scarce. The small number of amphipods from the last two samplings is accompanied by the appearance in great numbers of *Tubifex* sp. in June 2023, followed by the domination of the genus in May 2024.

The environmental status calculated from the biological indices of the benthic invertebrate fauna shows that K1 and K2 are always in bad ecological status, according to all the indices used. The best results are reported on K0, where S reports result in good and moderate conditions. BI, BMWP, and ASPT were always in moderate condition, excluding November 2022 when BMWP reported poor status. The EPT has the lowest scores of all indices and was almost always in very poor condition and once in poor condition since June 2022 (Table 2). %EPT was below 1% in the first two samples taken and twice around 1.5% in the last two samples. The DOMN has the best results and lowest values in 2023 and 2024, respectively.

K3 does not "achieve" a good ecological status on any of the indices. The best one is "moderate". S does not reach a moderate state only in the samples taken in 2024 when worms of the genus *Tubifex* sp. are the overdominant taxa. These representatives of the family Tubificidae in Greek rivers influenced by DPs were recorded at a site immediately after the point of discharge and not at those further away (Karadima et al., 2010).

BI was also in the "moderate" spectrum always, except for 2024. BMWP rates the site condition as poor in 3 out of 4 samples taken.

3.3. Physicochemical parameters

The temperature regime in the river section was monitored solely during samplings. This methodological shortcoming prevents the drawing of clear conclusions about thermal alteration. Nevertheless, warming of the river between K0 and K1 was observed. The greater temperature difference was registered in June 2023, when T was 2.3 °C higher at K1, compared to K0. The second most pronounced difference was 0.9 °C, from April 2022. The other 3 measurements registered differences between 0.2 and 0.4 °C (Table 3). The probable reason for the warming from K0 to K1 could be the different intensity of discharging wastewater and the lack of shading from a tree cover at K1 combined with the low velocity of the stream.

DO from K0 to K3 varies between 3.8 and 11.4 mg/L, with the lowest value recorded at K3 and the highest at K0. These results are above and under the typical concentrations for the R11 river type (6.2 – 9.4 mg/L) (RBMP, 2016). Only two measurements from November 2022 at K2 and K3 are in the moderate ecological status specter. In most samplings, the oxygen content was better than the GES scale results (Table 3). Average and interquartile range (IQR) scores in the DO at all sites showed a steady decrease in the parameter downstream from K0. After the third kilometer at K3, DO partly restored its content (Figure 3).

Table 1. Taxonomic distribution of macroinvertebrates indicated by sampling sites and events.

| | K0 | | | K1 | K2 | K3 | | | | |
|--|------------|------------|------------|------------|-----------|-----------|------------|-------------|------------|------------|
| | Jun 22 | Nov 22 | Jun 23 | May 24 | Jun 23 | Jun 23 | Jun 22 | Nov 22 | Jun 23 | May 24 |
| Oligochaeta | 9 | 440 | 78 | | | | | 415 | | |
| <i>Tubifex sp</i> | | | 10 | | | | | | 112 | 265 |
| Hirudinidae | | | | | | | | | | |
| Glossiphoniidae | | | | | | | 1 | | | |
| Gastropoda | | | | | | | | | | |
| Hydrobiidae | | | 1 | 8 | | | 2 | | | |
| Planorbidae | 5 | | 3 | 1 | | | 2 | | 4 | |
| Clausidae | 3 | | | | | | 1 | 1 | | |
| Chondrinidae | | | | 2 | | | | | | |
| Bivalvia | | | | | | | | | | |
| <i>Sphaerium sp.</i> | | | | 36 | | | 2 | 3 | 2 | |
| Malacostraca | | | | | | | | | | |
| <i>Assellus sp</i> | 2 | | | | | | | | | |
| Gammaridae | 736 | 192 | 12 | 210 | | | 22 | 546 | 2 | 10 |
| Ephemeroptera | | | | | | | | | | |
| Baetidae | 4 | | | 7 | | | | | | |
| Trichoptera | | | | | | | | | | |
| Hydropsychidae | | | 1 | | | | | | | |
| Philopotamidae | 2 | 1 | | | | | | | | |
| Megaloptera | | | | | | | | | | |
| Sialidae | | 6 | | 12 | | | | | | |
| Coleoptera | | | | | | | | | | |
| Colymbetinae | 2 | 2 | 15 | 12 | | | | | | |
| Dytiscinae | | | | 2 | | | | | | |
| Elmidae | | | | 1 | | | | | | |
| Gyrinidae | | | | 2 | | | | | | |
| Helodidae | | 5 | | | | | | | | |
| Odonata | | | | | | | | | | |
| Aeshnidae | | | | 1 | | | | | | |
| Calopterygidae | | | | | | | 5 | | | |
| Diptera | | | | | | | | | | |
| Athericidae | | | | | | | | | | 1 |
| Chironomidae | 4 | | 22 | 82 | | | 836 | 76 | 551 | |
| <i>Chironomus sp.</i> | | | | 10 | 10 | 56 | | 29 | | |
| Simuliidae | | | | | | | 40 | 1 | 90 | |
| Tabanidae | 2 | 16 | 2 | | | | 1 | | | |
| Psychodidae | | | | | | | | | 3 | |
| Tipulidae | | | | 2 | | | | | | |
| Heteroptera | | | | | | | | | | |
| Gerridae | 2 | | | | | | | | | |
| <i>Nepa cinerea</i> | 20 | | | | | | | | | |
| <i>Ranatra linearis</i> | | 5 | 2 | | | | | | | 20 |
| Abundance | 791 | 667 | 146 | 388 | 10 | 56 | 912 | 1071 | 765 | 295 |
| Total number of taxa for sampling site | | 26 | | | 1 | 1 | | 16 | | |

The pH ranged from 7.2 to 8.02. Again, the lowest values were from K0, and the highest from K3, remaining always in the GES spectrum (Table 3). The average values of the parameter at K0 are higher than those at K1 and K2 and lower than those at K3. The highest fluctuations were detected at K2 (Figure 3).

EC was a parameter varying in wide limits

from 625 to 3760 $\mu\text{S}/\text{cm}^2$. Almost every *in situ* measurement was better than the GES range, excluding K1 in June 2023 when an extremely high parameter value was reported, and at K3 in November 2022 (Table 3). Longitudinally EC was slightly increasing its mean and IQR values from K0 to K3 (Figure 3).

Table 2. Results of the biological indices represented by the color pattern of the WFD. Green – GES, good ecological status. Yellow – moderate ecological status. Orange - poor ecological status. Red - bad ecological status. K0/06.22 – K0 results from June 2022. K3/06.22 – K3 results from June 2022, etc.

| | S | BI | BMWP | ASPT | EPT | %EPT | DOMN |
|------------|----|-----|------|------|-----|------|------|
| K0/06.2022 | 12 | 3 | 47 | 3.92 | 2 | 0.76 | 0.86 |
| K0/11.2022 | 8 | 2.5 | 36 | 4.5 | 1 | 0.15 | 0.52 |
| K0/06.2023 | 10 | 2.5 | 43 | 4.3 | 1 | 1.43 | 0.2 |
| K0/05.2024 | 15 | 3 | 61 | 4.07 | 1 | 1.51 | 0.27 |
| K1/06.2022 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| K1/11.2022 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| K1/06.2023 | 1 | 1 | 2 | 2 | 0 | 0 | 1 |
| K1/05.2024 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| K2/06.2022 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| K2/11.2022 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| K2/6.2023 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| K2/5.2024 | 1 | 1 | 2 | 2 | 0 | 0 | 1 |
| K3/06.2022 | 10 | 2.5 | 41 | 4.1 | 0 | 0 | 0.84 |
| K3/11.2022 | 8 | 2.5 | 27 | 3.38 | 0 | 0 | 0.42 |
| K3/06.2023 | 8 | 2.5 | 27 | 3.38 | 0 | 0 | 0.55 |
| K3/05.2024 | 3 | 2 | 12 | 4 | 0 | 0 | 0.81 |

The parameters analyzed in the laboratory related to biogenic elements such as nitrogen and phosphorus are presented with their molecular forms (NO₂, NO₃, etc.) and not by their atomic forms (NO₂-N, NO₃-N). That way of representing the results was chosen, even though the atomic representation corresponds to certain ES, according to Ordinance H-4/2012 (Government of Bulgaria, 2012) because the available reagents are intended precisely to measure the

molecular concentrations.

Nitrites of K0 recorded values ranging from 0.03 to 1.15 mg/L, with the maximum and minimum values recorded in June and November 2022, respectively, and the average value for the site of 0.34 mg/L. At K1, the average value was three times lower - 0.11 mg/L. The maximum and minimum values were recorded in April 2022 and November 2022, respectively.

Table 3. Results of the physicochemical parameters represented by the color pattern of the WFD. Blue – high ecological status. Green – GES, good ecological status. Yellow – moderate ecological status. K0/04.22 – K0 results from April 2022. K1/04.22 – K1 results from April 2022, etc.

| | T °C | DO mg/L | pH | EC µS/cm ² | NO ₂ mg/L | NO ₃ mg/L | NH ₃ mg/L | PO ₄ mg/L | SO ₄ mg/L |
|------------|---------|------------|------|--------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| K0/06.2022 | 11.5 | 8.5 | 7.7 | 683 | 0.09 | 10.4 | 0.01 | 0.15 | 35 |
| K0/11.2022 | 13 | 8.9 | 7.77 | 705 | 0.03 | 0.8 | 0.11 | 1.62 | 35 |
| K0/06.2023 | 11.3 | 7.6 | 7.56 | 689 | 1.15 | 16.9 | 0.46 | 0.34 | 40 |
| K0/05.2024 | 14.6 | 11.4 | 7.54 | 660 | 0.37 | 7.8 | 0.0175 | 0.2 | 35 |
| K1/06.2022 | 13.8 | 9.1 | 7.56 | 625 | 0.036 | 6.16 | 0.006 | 0.58 | 36.25 |
| K1/11.2022 | 12.4 | 8 | 7.53 | 720 | 0.26 | 0 | 1.58 | 0.93 | 30 |
| K1/06.2023 | 13.2 | 8.5 | 7.78 | 754 | 0.06 | 23.63 | 0.11 | 0.79 | 30 |
| K1/05.2024 | 11.7 | 5.8 | 7.48 | 745 | 0.04 | 5.85 | 1.86 | 1.3 | 35 |
| K2/06.2022 | 16.9 | 6.7 | 7.3 | 3760 | 0.08 | 25.15 | 2.89 | 1.59 | 30 |
| K2/11.2022 | 14 | 7.25 | 7.2 | 690 | 0.11 | 0.87 | 0.32 | 1.15 | 31.25 |
| K2/6.2023 | 12 | 7 | 7 | 781 | 0.08 | 3.55 | 1.3 | 1.28 | 30 |
| K2/5.2024 | 13.8 | 8 | 7.91 | 707 | 0.19 | 7.1 | 0.013 | 2.5 | 25 |
| K3/06.2022 | 11.1 | 4.4 | 7.51 | 833 | 0.02 | 5.06 | 0.76 | 1.57 | 35 |
| K3/11.2022 | 14.5 | 5.5 | 7.27 | 751 | 0.02 | 4.73 | 1.88 | 1.48 | 55 |
| K3/06.2023 | 14.2 | 6.23 | 7.64 | 803 | 0.023 | 4.93 | 0.01 | 1.7 | 36.25 |
| K3/05.2024 | 14 | 9.8 | 8.02 | 756 | 0.28 | 9.83 | 0.106 | 0.66 | 50 |
| K0/06.2022 | 11.4 | 3.8 | 7.7 | 971 | 0.02 | 11.03 | 0.11 | 1.54 | 50 |
| K0/11.2022 | 15.1 | 7.3 | 7.57 | 800 | 1.15 | 19.73 | 0.06 | 0.58 | 55 |
| K0/06.2023 | 12.6 | 6.97 | 7.8 | 890 | 0.096 | 0 | 0.056 | 2.5 | 51.66 |

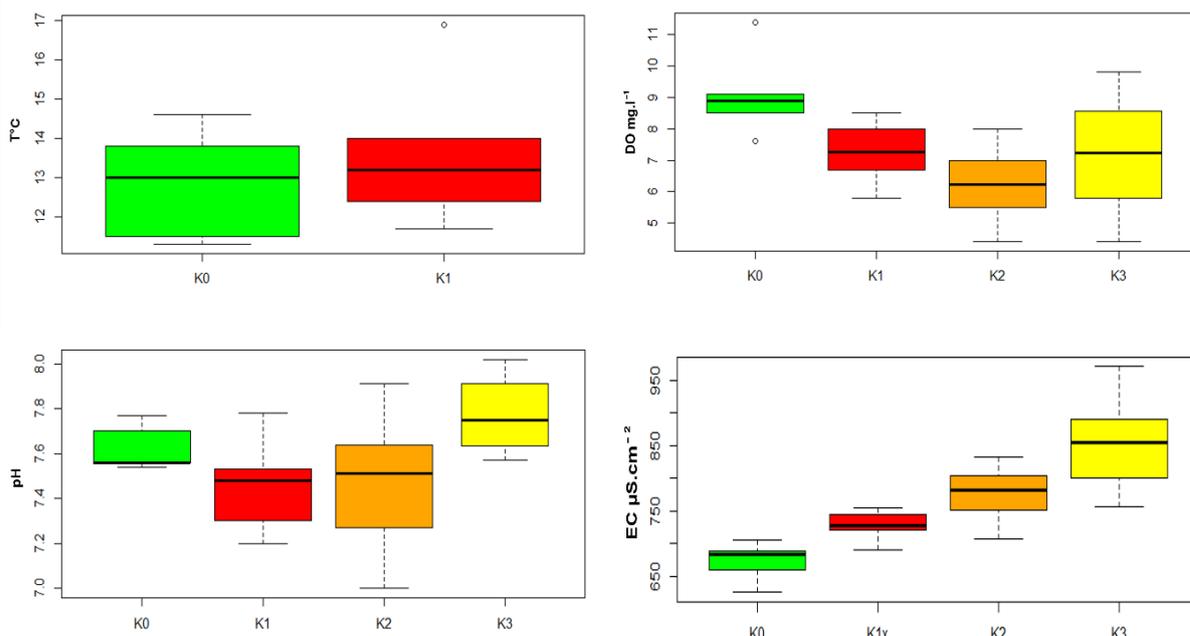


Figure 3. Boxplots of *in situ* measured parameters. K1 with "x" marks that EC scores on this site are specific because the extremely high value from June 2023 was not used for visualization by boxplot.

K2 demonstrated a further decrease in NO_2 and recorded an average value of 0.066 mg/L. June 2022 has the highest reported values for the parameter. The lowest was measured during the last 3 samplings. K3 registered an average value for NO_2 of 0.39 mg/L, which is also the highest concentration for this form of nitrogen in the studied river reach (Figure 4). The maximum value is from June 2023 and the minimum is from November 2022 (Table 3).

Mean and IQR scores for the studied period for all sampling sites reveal a tendency of nitrites to decrease in the water column downstream from the DP for 1.5 km of the river section, followed by a sharp increase at K3 (Figure 4). This longitudinal alteration throughout sites is probably sustained by the abundant primary producers at K1 and K2, presented by sewage fungus which can use dissolved inorganic nitrogen, including nitrites as a food source. An example of this is *E. coli* which can utilize hyponitrite as a nitrogen source (Nicholas, 1963) which was quite abundant in the mixed sample from K1 and K2, taken in November 2022.

Ammonium at K0 had scores between 0.006 and 0.46 mg/L. The maximum was recorded in November 2022 and the minimum in May 2024. The mean value for the site was 0.12 mg/L. At K1, the average value was more than 11 times higher – 1.35 mg/L. The higher and lower content were from June 2023 and June 2022, respectively. After the sharp saturation of NH_3 in K1, at K2 this biogenic element dropped to an average value of 0.79 mg/L. June 2023 and June 2022 again had the highest and lowest concentrations for the parameter. K3 registered the lowest average value for the river reach of 0.083 mg/L, with the lowest result from June 2023 and

the highest from November and June 2022. IQR and average results for the whole river stretch reveal a clear longitudinal gradient specific to K1, with a sharp increase of NH_3 followed by a steady decrease in a downstream direction towards K3 (Figure 4). This tendency is quite logical since nitrogen compounds such as ammonium ions can be found in dairy effluent (Slavov, 2017) and because ammonia is easily assimilated in freshwaters (Doychev & Taneva, 2025). Phosphates at K0 were with the lowest mean values for the studied river reach (0.578 mg/L), registering a maximum score (1.62 mg/L) from June 2022 and a minimum one (0.15 mg/L) from April 2022. K1 shows augmentation considering this parameter with an average result of 1.15 mg/L, with the highest scores from June 2023 (1.59 mg/L) and the lowest from June 2022 (0.79 mg/L). Towards K2 the augmentation continues and reaches an average phosphate concentration of 1.706 mg/L. The greatest (2.5 mg/L) and the lowest (1.28 mg/L) quantities were registered from June and April 2022, as at K0. At K3 the same maximum concentration was reached in the last sampling event from May 2024, but the minimum dropped twofold compared to K2, to 0.66 mg/L (Table 3). The average value decreased by 22.5% to 1.32 mg/L. Considering means and IQR values, a clear longitudinal augmentation of phosphates towards K2 and K3 is visible. The maximum scores at K2 and K3 are equivalent, but the minimum scores are lower at the last sampling site (Figure 4).

Sulfates at K0 were with concentrations between 35 and 40 mg/L and a mean score of 36.3 mg/L. At K1 a slight reduction was registered

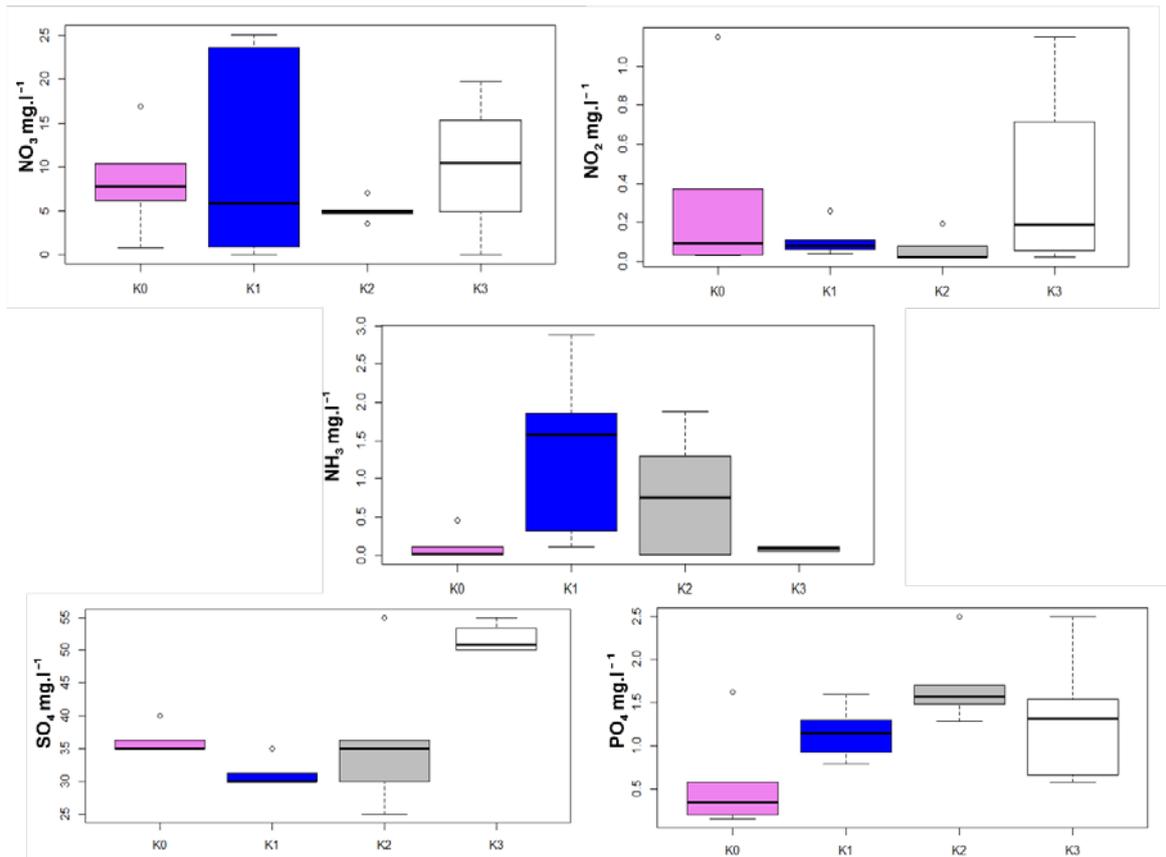


Figure 4. Boxplots of physicochemical parameters.

because concentrations varied from 30 and 35 mg/L with an average value for the parameter of 31.3 mg/L. At K2, SO₄ registered the same average value as on K0, but the amplitudes were greater. The maximum one was 15 mg/L higher and was measured in June 2023, the minimum one was with 10 mg/L lower and was from June 2022 (Table 3). K3 had the highest average value (51.7 mg/L) for this salinity parameter (European Commission, 2024) for the entire river reach (Figure 4).

Table 4. Satellite data results. K0/03-04.22 – Polygon results for the 2022 March-April period. K0/04-05.24 – Polygon results for the 2024 April-May period, etc.

| | NDVI | NDMI |
|-------------|---------|----------|
| K0/03-04.22 | 0.35198 | -0.02194 |
| K0/05-06.22 | 0.68184 | 0.27933 |
| K0/10-11.22 | 0.39565 | -0.12152 |
| K0/05-06.23 | 0.60196 | 0.23657 |
| K0/04-05.24 | 0.63037 | 0.24296 |

3.4. Satellite data

The polygon from which the data about NDVI and NDMI mean scores were extracted had a perimeter of 2208 m (Figure 1). NDVI and NDMI registered higher values for the warmer periods before water sampling events and lower ones for the March-April

and October-November periods. NDVI and NDMI reached their maximum values in the 2022 May-June period and their minimum from the 2022 March-April period (Table 4).

3.5. Interrelationships

3.5.1. PCQEs dependency from normalized differences of vegetation and moisture indices

MFA results that include PCQE scores from K0 and mean values for NDVI and NDMI for 1 month before water samplings showed a great difference between the contribution of the first two principal components. Dim 1 had a 61.7% contribution and within the principal component are well observed the negative correlations between the group “Sentinel2” with all nitrogen-containing parameters and sulfates. Particularly, the very strong negative correlation between nitrates and normalized difference of vegetation and moisture indices and a strong one with nitrites, ammonia, and sulfates (Figure 5). Nevertheless, solely NO₃ from the “PCQEs” group had a contribution greater than the average one (Figure 6) and therefore are of great importance for the variance (Kassambara, 2017). The “dimdesc” function confirms this since only NDMI, NDVI and NO₃ are statistically significant within Dim 1 (Table 5).

Dim 2 recorded a 20.2% contribution to the variance and strong positive correlation coefficients between EC, pH, and PO₄ (Figure 5). Solely the pH is statistically significant (Table 5).

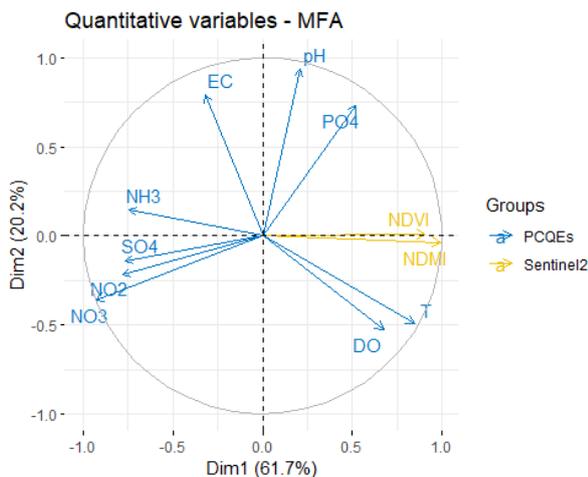


Figure 5. MFA correlation circle that includes PCQEs scores from K0 and NDVI/NDMI values from the polygon.

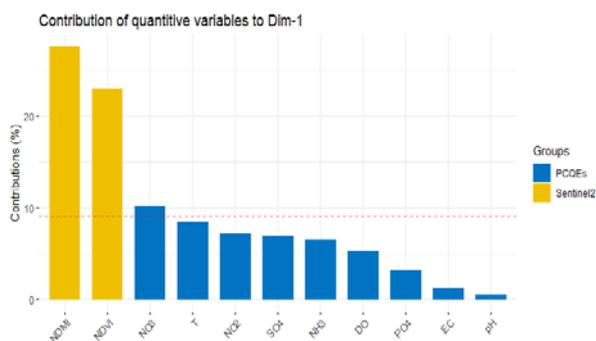


Figure 6. Bar plot demonstrating the contribution of the variables for “PCQEs” and “Sentinel2” groups. The red dotted line shows the average contribution for the dimension.

Table 5. Statistically significant parameters from “PCQEs” and “Sentinel2” groups.

| | correlation | p-value |
|-----------------|-------------|---------|
| Dim 1 | | |
| NDVI | 0.99156 | 0.00093 |
| NDMI | 0.90503 | 0.03463 |
| NO ₃ | -0.93038 | 0.02182 |
| Dim 2 | | |
| pH | 0.93671 | 0.01893 |

The condition of the watershed area used for remote data extraction demonstrated increased plant cover by the higher NDVI and NDMI values during warmer months (Table 4). This is related to the increased physiological activity of the plants that results in increased uptake of water and chemical compounds such as nitrogen and phosphorus-containing parameters, especially in agricultural areas

like the studied one (Griffith et al., 2002). In our case, the most significantly correlating PCQEs were nitrates, which leach from the river surroundings mostly in the months with bare soil cover, when fertilizers are applied.

3.5.2. Macroinvertebrates condition dependency from PCQEs

MFA shows that Dim 1 is responsible for 33.4% of the variation and Dim 2 for 18.6%. All biological indices are grouped around Dim 1. Figure 7 also demonstrates that DOMN correlates positively with NH₃ and PO₄, while the rest of the “invertebrates” variables had strong negative correlations with the just mentioned PCQEs within Dim 1. In addition, in the first principal component DO and pH correlate positively with ASPT, BI, S, BMWP, %EPT, EPT, NH₃, and PO₄. Principal component 2 shows interrelations only between physical and chemical parameters particularly the strong positive correlation for EC, T, and NO₃.

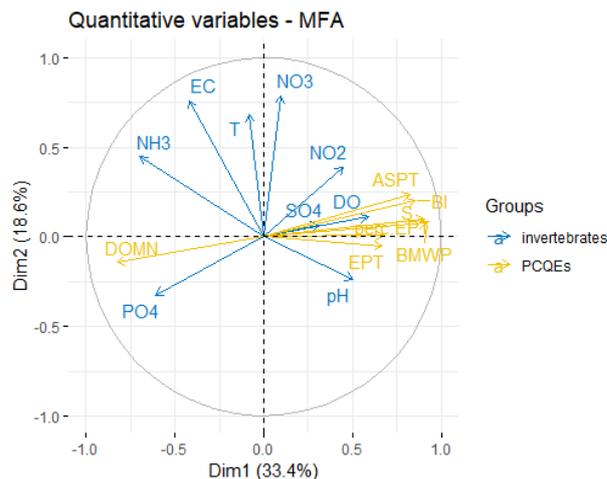


Figure 7. MFA correlation circle that includes PCQE scores and invertebrate indices scores from all sites.

The variable with the greatest contribution for Dim 1 is NH₃. Contribution close to the average for the dimension was registered from 3 more physicochemical parameters along with 5 biological indices (Figure 8). Those variables are the most important for the variance (Kassambara, 2017) and are statistically significant (Table 6).

The highest positive and negative correlation coefficients (Table 6) are between the first two most contributing parameters (Figure 8) and therefore ammonia and BMWP explain the greatest part of the variance. Of all “invertebrates” variables the greatest sensitivity to organic pollution and the changing nutrient contents within this heavily polluted river reach was registered by BMWP.

From the measured PCQEs, NH₃ had the greatest

influence on BMWP (Table 6), which is a parameter that could acutely affect invertebrate tissues through oxidative or physiological stress and by reducing the capability of growing and reproducing (Doychev & Taneva, 2025).

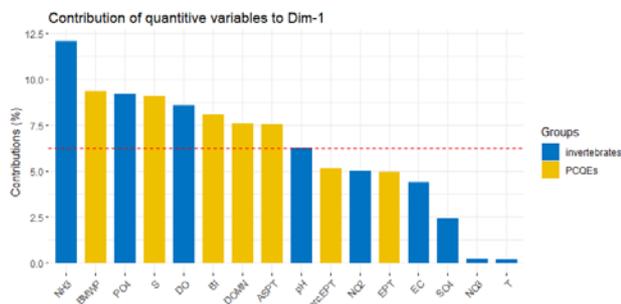


Figure 8. Bar plot demonstrating the contribution of the variables for “invertebrates” and “PCQEs” groups. The red dotted line shows the average contribution for the dimension.

Table 6. Statistically significant parameters from “invertebrates” and “PCQEs” groups.

| | correlation | p-value |
|-----------------|-------------|-----------|
| Dim 1 | | |
| BMWP | 0.91950 | 2.625e-08 |
| S | 0.90601 | 9.346e-08 |
| BI | 0.85344 | 3.387e-06 |
| ASPT | 0.82508 | 1.376e-05 |
| %EPT | 0.67907 | 1.388e-03 |
| EPT | 0.66953 | 1.715e-03 |
| DO | 0.59013 | 7.819e-03 |
| pH | 0.50259 | 2.830e-02 |
| PO ₄ | -0.61019 | 5.529e-03 |
| NH ₃ | -0.69948 | 8.585e-04 |
| DOMN | -0.82738 | 1.240e-05 |
| Dim 2 | | |
| NO ₃ | 0.78764 | 6.243e-05 |
| EC | 0.76027 | 1.581e-04 |
| T | 0.68359 | 1.252e-03 |

The phosphorus-containing variable is the second most contributing PCQE in Dim 1 (Figure 8) that registers a strong negative correlation with BMWP, S, BI, and ASPT (Figure 8, Table 6). This confirms that the river stretch and in-stream fauna are seriously affected by the high concentrations of PO₄ and thus confirms the significance of one of the main reasons for water bodies' degradation and biodiversity reduction on a global scale, the phosphorus enrichment (Everall et al., 2019).

Another interesting interrelationship is the strong positive correlation between phosphates and DOMN (Figure 8, Table 6), bearing in mind that the highest recorded values of phosphates at K3 were reported with the largest number of individuals of the genus *Tubifex sp.* and their over-domination at the site during the last sampling event (Table 1). These worms can mobilize phosphorus from accumulated organic sediments

(Hendriks et al., 2024) and cause immediate negative effects on aquatic organisms, since only 3 taxa were found at K3 in May 2024.

4. CONCLUSIONS

Our research reflects spatial and temporal changes in an agricultural river reach subjected to multiple stressors. In the first place, we confirmed the chronic diffusive pollution with inorganic nitrogen from the arable surroundings by relying on proven indices such as NDVI and NDMI, using the Sentinel-2 LMA dataset. This influx from adjacent areas provides a continuous food source for microorganisms such as *E. coli* (Nicholas et al., 1963) and ensures a steady flow of nutrients even when there is no wastewater discharge. Thus, agricultural practices provide a better environment for bacterial populations and may indirectly contribute to prolonging the health risk.

The single microbiological analysis revealed the presence of abundantly developed health-threatening bacteria within 1.5 km of the riverbed downstream from the DP. We also suggest that some of the longitudinal fluctuations in nitrites and nitrates are due to the abundant presence of *Pseudomonas spp.* and *Escherichia spp.* and therefore the regulation of their populations is important for the good physicochemical condition of the river.

Other specific longitudinal gradients related to average and IQR values of main biogenic elements were also established. Surprisingly in some cases, they are related to the augmentation of the scores and the concentrations in a downstream direction towards K3 and were not assimilated but were saturated in the water column. An example of such a gradient is that of electrical conductivity, the increase of which is related to the spread of more pollution-tolerant invertebrates in Bulgarian rivers (Varadinova et al., 2023).

We suggested the character of some of the water quality gradients by the found bacteria and invertebrate taxa specifics. Nevertheless, a furthermore detailed investigation will be needed if disentangling the mechanism dynamics of temporal self-purification is searched.

This is the first ecological investigation conducted in any of the 3 water bodies in the Kriva Reka River. The study shows the incapability of the system to recover the invertebrates in 3.3 km and to maintain even moderate status for the studied 3-year period.

The lack of a functioning water purification plant in the DP in Kriva Reka Village is unacceptable considering the water quality, the aquatic fauna ecological status, and the presence of health-threatening microorganisms in large quantities. In addition, the fact that our country published the projects of the third stage

of the river basin management plans (RBMPs) (2021 - 2027), just in 2024, although the finalization of the application of the RBMPs coincides with the deadline given by the WFD, particularly to achieve GES for all surface water bodies (European Commission, 2019), gives additional weight to the problem and points out the need of urgent measures.

Acknowledgments

This research is supported by the Bulgarian Ministry of Education and Science for the second stage of the National Program "Young Scientists and Postdoctoral Students - 2" and by Konstantin Preslavsky University of Shumen, Bulgaria, under Projects No. RD-08-108/30.01.2024 and No. RD-08-126/01.02.2024.

REFERENCES

- Ahmad, T., Aadil, R.M., Ahmed, H., ur Rahman, U., Soares, B.C.V., Souza, S.L.Q., Pimentel, T.C., Scudino, H., Guimarães, J.T., Esmerino, E.A., Freitas, M.Q., Almada, R.B., Vendramel, S.M.R., Silva, M.C. & Cruz, A.G., 2019. *Treatment and utilization of dairy industrial waste: A review*. Trends in Food Science & Technology 88, 361–372, <https://doi.org/10.1016/j.tifs.2019.04.003>.
- Atnafie, B., Paulos, D., Abera, M., Tefera, G., Hailu, D., Kasaye, S. & Amenu, K., 2017. *Occurrence of Escherichia coli O157:H7 in cattle feces and contamination of carcass and various contact surfaces in abattoir and butcher shops of Hawassa, Ethiopia*. BMC Microbiology, 17(1), 24, <https://doi.org/10.1186/s12866-017-0938-1>.
- Cheshmedjiev, S., Soufi, R., Vidinova, Y., Tyufekchieva, V., Yaneva, I., Uzunov, Y. & Varadinova, E., 2011. *Multi-habitat sampling method for benthic macroinvertebrate communities in different river types in Bulgaria*. Water Research and Management, 1(3), 55-58.
- Cheshmedjiev, S. & Varadinova, E., 2013. *Bottom invertebrates*. In: D. Belkinova, G. Gecheva, J. Uzunov (Eds), *Biological analysis and ecological assessment on the surface water types*. Paisiy Hilendarski University, Publishing House, Plovdiv. (in Bulgarian).
- Chu, H.J., Liu, C.Y. & Wang, C.K., 2013. *Identifying the Relationships between Water Quality and Land Cover Changes in the Tseng-Wen Reservoir Watershed of Taiwan*. International Journal of Environmental Research and Public Health, 10, 478-489, <https://doi.org/10.3390/ijerph10020478>.
- Demirel, B., Yenigun, O. & Onay, T.T., 2005. *Anaerobic treatment of dairy wastewaters: a review*. Process Biochemistry, 40, 2583–95, <https://doi.org/10.1016/j.procbio.2004.12.015>.
- Doychev, D.D., 2023. *Longitudinal recovery gradient of macroinvertebrates during different hydrological scenarios in a downstream river reach*. Journal of Limnology, 82, 2125, <https://doi.org/10.4081/jlimnol.2023.2125>.
- Doychev, D.D., & Taneva, L.R., 2025. *An unpolluted regulated stream and its recovery gradient dependency from environmental variables*. Limnetica, 44(1), 000-000, <https://doi.org/10.23818/limn.44.11>.
- European Commission, 2005. Common implementation strategy for the Water Framework Directive (2000/60/EU). *Guidance Document No. 13. Overall approach to the classification of ecological status and ecological potential*. Available from: [https://circabc.europa.eu/sd/a/06480e8727a641e6b1650581c2b046ad/Guidance%20No%2013%20%20Classification%20of%20Ecological%20Status%20\[WG%20A\].pdf](https://circabc.europa.eu/sd/a/06480e8727a641e6b1650581c2b046ad/Guidance%20No%2013%20%20Classification%20of%20Ecological%20Status%20[WG%20A].pdf).
- European Commission, 2019. Commission staff working document. *Fitness Check of the Water Framework Directive, Groundwater Directive, Environmental Quality Standards, Directive and Floods Directive*. Brussels, 10.12.2019, SWD, 439 final. Available from: https://commission.europa.eu/publications/fitness-check-water-framework-directive-and-floods-directive_en#details.
- European Commission, Joint Research Centre, Kelly, M., Teixeira, H., Lyche Solheim, A., Free, G., Phillips, G., Salas Herrero, M.F., Kolada, A., Varbiro, G. & Poikane S. 2024. *Physico-chemical criteria to support Good Ecological Status in Europe*. Publications Office of the European Union, Luxembourg, https://data.europa.eu/doi/10.2760/355815_JRC136407.
- European Environment Agency (EEA), 2018. *European waters – Assessment of status and pressures 2018*. EEA Report No 7/2018. EEA, Copenhagen. Retrieved from: <https://www.eea.europa.eu/publications/state-of-water>.
- Everall, N.C., Johnson, M.F., Wood, P., Paisley, M.F., Trigg, D.J. & Farmer, A., 2019. *Macroinvertebrate community structure as an indicator of phosphorus enrichment in rivers*. Ecological Indicators, 107, 105619, <https://doi.org/10.1016/j.ecolind.2019.105619>.
- Gao, B.C., 1996. *NDWI A Normalized Difference Water Index for Remote Sensing of Vegetation Liquid Water From Space*. Remote Sensing of Environment, 58, 257-266, [https://doi.org/10.1016/S0034-4257\(96\)00067-3](https://doi.org/10.1016/S0034-4257(96)00067-3).
- Government of Bulgaria, 2012. *Ordinance H-4 from 14 of September 2012 for surface water characterization*. (in Bulgarian). Available from: [Naredba H-4.pdf](https://www.naredba.bg/Naredba-H-4.pdf) (government.bg).
- Griffith, J.A., Martinko, E.A., Whistler, J.L. & Price, K.P., 2002. *Interrelationships Among Landscapes, NDVI, and Stream Water Quality in the US Central Plains*. Ecological Applications, 12(6), 1702-1718, Available at: http://aquila.usm.edu/fac_pubs/3442.
- Guareschi, S., Laini, A. & Sánchez-Montoya, M.M., 2017. *How do low abundance taxa affect river biomonitoring? Exploring the response of different macroinvertebrate-based indices*. Journal of Limnology, 76(s1), 9-20, <https://doi.org/10.4081/jlimnol.2016.1516>.
- Gümüş, İ., 2023. *Use of vermicompost to improve soil properties and spinach growth in the soil affected by wind erosion*. Carpathian Journal of Earth and Environmental Sciences, 18(2), 245–253, <https://doi.org/10.26471/cjees/2023/018/255>.

- Hawkes, H.A.**, 1998. *Origin and development of the biological monitoring working party score system*. *Water Research*, 32, 964–968, [https://doi.org/10.1016/S0043-1354\(97\)00275-3](https://doi.org/10.1016/S0043-1354(97)00275-3).
- Hendriks, L., van der Meer, T.V., Kraak, M.H.S., Verdonschot, P.F.M., Smolders, A.J.P., Lamers, L.P.M. & Veraart A.J.**, 2024. *Sludge degradation, nutrient removal and reduction of greenhouse gas emission by a Chironomus-Azolla wastewater treatment cascade*. *PLoS ONE*, 19(5), e0301459, <https://doi.org/10.1371/journal.pone.0301459>.
- Hieu, N.V., Lien, B.T. & Vinh, N.V.**, 2016. *Using macro-invertebrates as bio-indicator for assessment water quality of bodies in Ngoc Thanh Commune, Phuc Yen District, Vinh Phuc Province*. *VNU Journal of Science: Natural Sciences and Technology*, 32(1S), 56-62.
- Jamieson, R.C., Gordon, R.J., Tattrie, S.C. & Stratton, G.W.**, 2003. *Sources and Persistence of Fecal Coliform Bacteria in a Rural Watershed*. *Water Quality Research Journal of Canada*, 38(1), 33–47.
- Karadima, C., Theodoropoulos, C., Rouvalis, A., & Iliopoulou-Georgudaki, J.**, 2010. *Ecological risk assessment of cheese whey effluents along a medium-sized river in southwest Greece*. *Journal of Environmental Science and Health, Part A*, 45(6), 775–781, <https://doi.org/10.1080/10934521003651614>.
- Kassambara, A.**, 2017. *Practical Guide to Principal Component Methods in R*. Edition 1, <http://www.sthda.com>.
- Kucuk, S. & Alpbaz, A.**, 2008. *Water quality and protection: environmental aspects. The impact of organic pollution on the Kirmir Creek and Sakarya River in Turkey*. *Water Resources*, 35(5), 617–624, <https://doi.org/10.1134/S0097807808050102>.
- Mazari, H.E., Amina Meliani, A., Berkat, S., Aliane, S., Rachid Djibaoui, R., & Boudroua, K.**, 2024. *Washing of heavy metal-contaminated soils using pyoverdine extracted from plant growth-promoting bacteria *Pseudomonas lactis* and *P. atacamensis**. *Carpathian Journal of Earth and Environmental Sciences*, 19(1), 169 – 178, <https://doi.org/10.26471/cjees/2024/019/288>.
- Moskova, G., Soufi, R. & Uzunov, Y.**, 2008. *Application of the EPT-index for ecological status assessment of the riverine water bodies within the basin of Kamchia river*. *International Journal Bioautomation*, 11, 73-79.
- Muthukrishnan, S., Lewis, G.P. & Andersen, C. B.** 2007. *Relations among land cover, vegetation index, and nitrate concentrations in streams of the Enoree River Basin, piedmont region of South Carolina, USA*. In: D. Sarkar, R. Datta, R. Hannigan (Eds), *Developments in Environmental Science*, Volume 5, Elsevier Ltd, [https://doi.org/10.1016/S1474-8177\(07\)05024-3](https://doi.org/10.1016/S1474-8177(07)05024-3).
- Nicholas, D.J.D.**, 1963. *The metabolism of inorganic nitrogen and its compounds in micro-organisms*. *Biological Reviews*, 38, 530-568.
- Porwal, H.J., Mane, A.V. & Velhal, S.G.**, 2015. *Biodegradation of dairy effluent by using microbial isolates obtained from activated sludge*. *Water Resources and Industry*, 9, 1–15, <https://doi.org/10.1016/j.wri.2014.11.002>.
- R Core Team.**, 2023. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, <https://www.R-project.org/>.
- Ragunath, B.V., Punnagaiarasi, A., Rajarajan, G., Irshad, A., Elango, A. & Manesh kumar, G.**, 2016. *Impact of Dairy Effluent on Environment — A review*. In: M. Prashanthi, R. Sundaram (Eds), *Integrated Waste Management in India. Environmental Science and Engineering*. Springer International Publishing Switzerland, https://doi.org/10.1007/978-3-319-27228-3_22.
- River Basin Management Plan (RBMP)**, 2016. *River basin management plan of the Black Sea Basin Directorate 2016–2021*. Decision 1107/29.12.2016 of the Council of Ministers (in Bulgarian). Available from: https://www.bsbd.org/bg/index_bg_5493788.html.
- Schürings, C., Globevnik, L., Lemm, J.U., Psomas, A., Snoj, L., Hering, D. & Birk, S.**, 2024. *River ecological status is shaped by agricultural land use intensity across Europe*. *Water Research*, 251, 121136, <https://doi.org/10.1016/j.watres.2024.121136>.
- Shete, B.S. & Shinkar, N.P.**, 2013. *Dairy Industry Wastewater Sources, Characteristics & its Effects on Environment*. *International Journal of Current Engineering and Technology*, 3(5), 1611-1615.
- Shivsharan, V.S., Wani, M. & Khetmalas, M.B.**, 2013. *Isolation of microorganisms from dairy effluent*. *British Microbiology Research Journal*, 3(3) 346-354, <https://doi.org/10.9734/BMRJ/2013/3445>.
- Simpson, E.H.**, 1949. *Measurement of Diversity*. *Nature*, 163, 608, <https://doi.org/10.1038/163688a0>.
- Slavov, A.K.**, 2017. *General Characteristics and Treatment Possibilities of Dairy Wastewater – A Review*. *Food Technology and Biotechnology*, 55(1), 14–28, <https://doi.org/10.17113/ft.b.55.01.17.4520>.
- Tarr, P.I.**, 1994. *Escherichia coli O157: H7: Overview of Clinical and Epidemiological Issues*. *Journal of Food Protection*, 57(7), 632-636, <https://doi.org/10.4315/0362-028X-57.7.632>.
- Tikariha, A. & Sahu, O.**, 2014. *Study of Characteristics and Treatments of Dairy Industry Waste Water*. *Journal of Applied & Environmental Microbiology*, 2(1), 16-22.
- Varadinova, E., Gecheva, G., Tyufekchieva, V. & Milkova, T.**, 2023. *Macrophyte- and Macrozoobenthic-Based Assessment in Rivers: Specificity of the Response to Combined Physico-Chemical Stressors*. *Water*, 15, 2282. <https://doi.org/10.3390/w15122282>.

Received: 15.12.2024

Revised: 14.01.2025

Accepted: 18.01.2025

Published: 20.01.2025