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Original Contribution

MORPHOLOGICAL AND HEMATOLOGICAL PARAMETERS OF *CARASSIUS GIBELIO* (PISCES: GYPRINIDAE) IN CONDITIONS OF ANTHROPOGENIC POLLUTION IN SOUTHERN BULGARIA. USE OF HEMATOLOGICAL PARAMETERS AS BIOMARKERS

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ABSTRACT
Basic morphological and hematological parameters were studied in adult, sexually mature individuals of *Carrasius gibelio* living in two rivers with varying degrees and different types of anthropogenic pollution (less disrupted site, domestic sewage pollution and heavy metal pollution) in Southern Bulgaria. The study was conducted on 30 female fish, age 3+ from each site. The results show that in *C. gibelio*, which inhabit the site with heavy metal pollution, the morphological parameters BW and FCF have the lowest statistically significant values. Specimens of *C. gibelio* from the river site with domestic sewage pollution have the following hematological profiles: erythrocytosis, leukocytosis and hyperchromia, differential leukocyte count: neutrophilia, eosinophilia, monocytosis and lymphocytopenia. Red cell indices (MCH, MCHC and MCV) display the highest statistically significant values. The *C. gibelio* specimens from the site with heavy metal pollution had the following hematological profiles: erythrocytosis, leukocytopenia and hypochromia, differential leukocyte count: neutrophilia, eosinophilia, monocytosis and lymphocytopenia. The lowest statistically significant values of red cell indices (MCH, MCHC and MCV) indicate anemic changes of microcytic and hypochromic type. The hematological parameters of *C. gibelio* are adequate biomarkers of the physiological state of animals and their habitats.

Key words: Prussian carp, morphological parameters, hematological parameters, domestic sewage pollution, heavy metal pollution, environmental bioindicators.

INTRODUCTION

At the end of the last century, and the beginning of the present century, anthropogenic pollution of the environment turned out to be a global ecological problem which affects all countries. Human activities have negative effects on both marine and fresh water ecosystems.

Fish are very sensitive to anthropogenic pollution and some of them have been widely used in toxicological studies as models to evaluate the health of aquatic ecosystems (1). Hematological parameters are widely used indicators of environmental stress in fish. A literature survey shows that most experiments studying the hematological parameters of several marine and fresh water fish have been conducted in labs under controlled conditions. The aim of these studies is to identify the changes in the values of various hematological parameters under the effect of different types of toxicants as well as under the effect of their different concentrations. The changes in the blood of various representatives of Teleostei fishes have been tested under the effects of toxic plant extracts (2), crude oil (3), pesticides (4), various heavy metals (5) or a combination of several heavy metals (6). There have been relatively few studies carried out in seas and rivers polluted by human activities (7-10), where xenobiotics mix with the characteristics of the environment. This mixture complicates the identification of the relations among the changes in the values of hematological parameters (in the physiology of fish

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respectively) and the toxicants present in the environment.

Currently, fishes of the genus *Carassius* (Cypriniformes: Cyprinidae), including goldfish, crucian carp and Japanese crucian carp are more and more widely used as test subjects in various ecotoxicological studies (11-14). These freshwater fish are distributed widely in around the Eurasian continent (15). They can live in an environment with low oxygen content, variable temperatures and high levels of anthropogenic pollution (16). Although their classification has not been well established due to their great variability in recent years, a growing belief has appeared in ichthyology, however controversial, that silver Prussian carp *Carassius gibelio* (Bloch, 1882) and crucian carp *Carassius carassius* (Linnaeus, 1758) have the status of separate species (17).

In southern Bulgaria *C. gibelio* is a very common species while *C. carassius* has significantly decreased in number and this species has even been included in the Bulgarian Red Data Book (18).

The present research has been inspired by the insufficient information about the values of basic morphological and hematological parameters and their fluctuation in the populations of *C. gibelio* inhabiting river ecosystems polluted by various types of toxicants. Its purpose is to illustrate the opportunities for using some value changes of the hematological parameters investigated in *C. gibelio* for assessing fishes' physical status, as well as for giving additional information about their role as biomarkers in biomonitoring system.

MATERIAL AND METHODS

Study Area

The research was performed in two rivers located in the southern part of the Republic of Bulgaria, the rivers Sazliyka and Topolnitsa.

The samples for our study were collected in the summer of 2012 at three sampling sites (for convenience labeled as 1, 2 and 3) located along the courses of the two rivers: the river the river Sazliyka in its upper reaches below the village of Rakitnitsa – Site 1, in its middle part in the region of Radnevo – Site 2 and the river Topolnitsa below the village of Poibrene, below where the river Medetska joins the Topolnitsa reservoir – Site 3 **(Figure 1).**

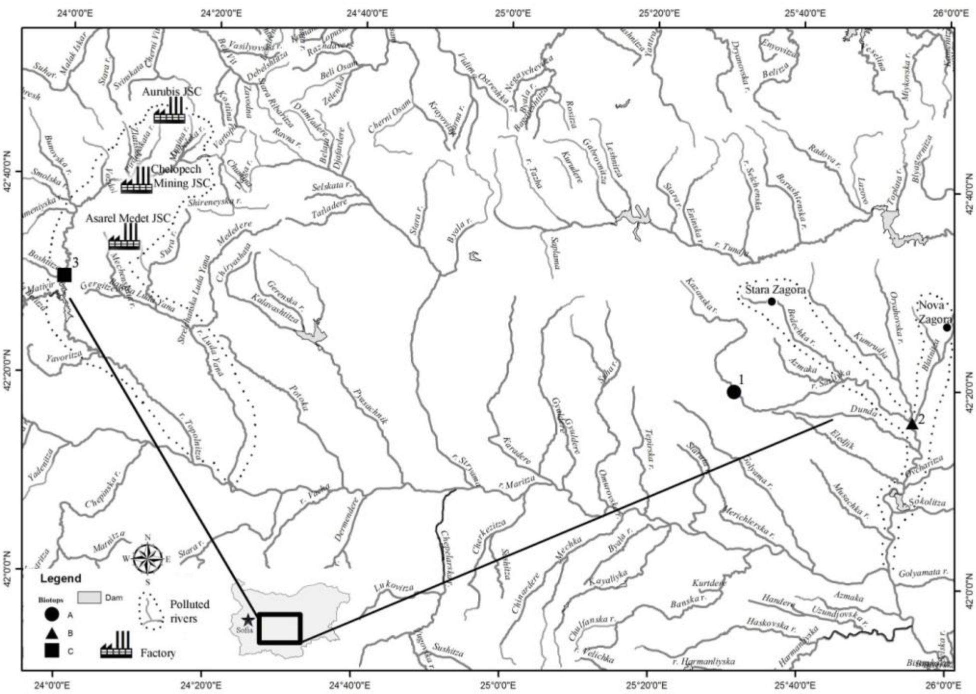

Figure 1. Geographical location of the water ecosystems studied

Legend: **Sites**: 1 – the river Sazliyka below the village of Rakitnitsa; 2 – the river Sazliyka below Radnevo; 3 – the river Topolnitsa below the village of Poibrene

Data from physicochemical analysis of the water ecosystems studied

The river Sazliyka is polluted mostly with domestic and industrial wastewaters from the towns Stara Zagora and Nova Zagora through its feeders: river Bedechka and river Blatnitsa. Two “hot spots”, which are of national importance, are marked along the river. The first one is located after the city of Stara Zagora near the confluence of river Bedechka and the second one is after the town of Radnevo at the confluence of river Blatnitsa. A 12 km stretch in the area of Stara Zagora is extremely affected, and the whole river course after Nova Zagora is also contaminated. After Nova Zagora, the treatment facilities are technologically outdated – there is no nitrogen and phosphorus removal stage, and they do not work effectively. Since April 2011, a modern waste water treatment plant was put into operation at the town of Stara Zagora. It has a three-stage treatment system – mechanical, biological and tertiary treatment for nitrogen and phosphorus. There are facilities for anaerobic sludge treatment managing for the entire load of river Bedechka. Hopefully, after the commissioning of Stara Zagora's waste water treatment plant, the environmental conditions in Sazliyka's upper course will improve. The pollutants of the river Topolnitsa are from Aurubis JSC, Bulgaria (former Pirdop) copper smelter and refinery, the Asarel Medet JSC, Bulgaria copper extracting and processing factory, and the landfill of Chelopech Mining JSC, Bulgaria **(Figure 1).**

Monitoring and control of the river Sazliyka and the river Topolnitsa surface water is performed by the National System for Environmental Monitoring (NSEM). The degree of pollution in sites 1, 2 and 3, and the nature of the pollutants have been determined on the basis of the data from the annual report of the Executive Environment Agency (http://eea.government.bg) on the condition of the environment (waters) in the Republic of Bulgaria for 2001-2012 , and the data from the physicochemical analysis of the water in the rivers Sazliyka and Topolnitsa for 2009-2012 from the reports by the Directorate for Water Management - East Aegean Region (http://www.bg-ibr.org) – **Table 1.**

Table 1. *Recent data on the sites at the time of the study (physicochemical analysis - surface water sample) The river Sazliyka below the village of Rakitnitsa - site 1 and below Radnevo - site 2; The river Topolnitsa below the village of Poibrene - site 3*

Parameters	**Units SI**	**Order №7/8.8.1986 categories**			**2012 Sites**		
		I (clean)	**II (slightly polluted)**	**III (highly polluted)**	**1**	**2**	**3**
pH	pH units	6.5-8.5	6.0-8.5	6.0-9.0	8.2	7.7	8.0
Temperature	°C	to 3° middle of the season			15.1	14.9	11.1
Insoluble substances	mg/dm^3	30	50	100	5	42.2	6.62
Electro conductivity	μS/cm	700	1300	1600	867	613	602
Dissolved oxygen	mgO_2/dm^3	6	4	2	7.23	5.3	5.55
Oxygenation	%	75	40	20	85.3	58.2	53
Biologic consummation of oxygen - BOD_5	mgO_2/dm^3	5	15	25	3.53	17.2*	3.3
Chemical consummation of oxygen - COD	mgO_2/dm^3	25	75	100	5.4	5.8	6.2
Nitrate ammonium $N\text{-}NH_4$	mg/dm^3	0.1	2	5	0.16	4.4*	0.02
Nitrate nitrogen $N\text{-}NO_3$	mg/dm^3	5	10	20	1.1	1.2	0.2
Nitrite nitrogen $N\text{-}NO_2$	mg/dm^3	0.002	0.04	0.06	0.002	0.2**	0.001
Orthophosphates P	mg/dm^3	0.2	1	2	0.022	0.433	0.302
Total nitrogen	mg/dm^3	1	5	10	1.95	5.3*	0.543
Total phosphorus - as P	mg/dm^3	0.4	2	3	0.28	0.42	0.32
Sulfates (SO_4^{2-})	mg/dm^3	200	300	400	24.32	54.8	212
Iron - total (Fe)	mg/dm^3	SKOS-0.1			< 0.001	< 0.001	0.20″
Manganese (Mn)	mg/dm^3	SKOS-0.05					0.33″
Copper (Cu)	mg/dm^3	SKOS-0.022					0.031″
Arsenic (As)	mg/dm^3	SKOS-0.25					0.003
Lead (Pb)	mg/dm^3	SKOS-0.0072					0.015″
Nickel (Ni)	mg/dm^3	SKOS-0.02					0.09″

Note:* – Above the admissible concentration limit for category II; ** – above the admissible concentration limit for category III; ″ – above SKOS; - assessment index: very poor condition

The site 1 is less disrupted and as such it is viewed as a "conventional control" in our study. There is no data about anthropogenic pollution for site 1 (2012 year, all 21 indicators are standard for Category I (clean), Category II (slightly polluted) and Category III (highly polluted) water basins, under Order №7/8.8.1986 (State Gazette, № 96. 12. 12. 1986). The two sites 2 and 3 are polluted. The main type of pollution in site 2 is domestic sewage, while site 3 is polluted with heavy metals.

Fish sampling
This study was carried out with the silver Prussian carp *C. gibelio*. The ichthyologic material was collected in August (Site 1: 14th August; Site 2: 20th August; Site 3: 27th August). The specimens were identified according to Kottelat and Freyhof, 2007 (16). The fishing was done with a fishing rod (permission certificate G/966611 issued on the 21st of October 2011 by the Executive Agency for Fisheries and Aquaculture, Republic of Bulgaria). The specimens were chosen selectively, and the selection aimed at working with larger, sexually mature specimens. Immediately after the fish had been caught, they were transported to the lab in containers full of water from the respective site, at permanent aeration. All analyses were done on the same day.

The Ethics Board for Experimental Animals, Faculty of Biology at Plovdiv University, approved the way the animals were handled and the methodology. All experiments were conducted in accordance with the national and international guidelines of the European Parliament and the Council for the Protection of Animals used for Scientific Purposes (19).

Morphological analysis
Specimens were measured (standard length, SL: measured from the anterior-most point of the snout or upper lip to the base of the median caudal rays) to the nearest 1 mm and weighed (total weight) to the nearest 0.1 g. Body weights were estimated using a digital weighing balance (KERN EMB 600-2, Germany). Scales were used for age estimation. For this purpose, scales were taken from the left side of the fish, above the lateral line, near the dorsal fin and stored in 33% ethanol. The preparations were examined under an Olympus SZ51 stereomicroscope. The sex of the fish was determined by macroscopic and microscopic investigations.

The health of the fish in this study was calculated according to Pauly, 1983 (20), thus: $K = (W/L^3) \times 10^2$, where W = body weight (in grams), L = standard length (in centimeters).

Hematological analysis
After fish capture, the procedures were conducted under laboratory conditions, on the same day, not more than two hours later, in accordance with the ethical standards for research work with live animals (21). The blood (0.20 ml) was taken by means of a cardiac ventricular puncture, using a small heparinized needle (20 mm length).

The erythrocyte (RBC) and leukocyte (WBC) count was determined according to the method of Wierord using a Burker chamber (22). For dilution a standardised Hayem solution was used for erythrocytes via Thoma pipettes, while for the leucocytes we used Turck's solution. Dilution was carried out 200 times for the erythrocyte count and 20 times for the leukocyte count. The hemoglobin concentration (Hb) was determined with the cyan-hemoglobin method by reading of absorbance at 540 nm with UV/Visible spectrophotometer (Eppendorf BioSpectrometer®, Germany). The packed cell volume PCV (or hematocrit value) was determined with heparinized hematocrit capillaries. Blood was centrifuged for 5 min at 3000 rpm constant-rotation (Eppendorf 5430R, Germany), in thin-walled capillary tubes by and the value obtained was read from the scale and recorded in L/L (23). The derivative hematological parameters (MCH - mean corpuscular hemoglobin, MCHC - mean corpuscular hemoglobin concentration, and MCV – mean corpuscular volume) were calculated mathematically from the results above, according to formulas Brown, 1980 (24). MCV was calculated by dividing hematocrit per liter of blood by total RBC count. The differential leukocyte count (Leukogram) was determined on the basis of 100 leukocytes per slide, using Shiling's microscopic method with Olympus stereo microscope (SZX16, resolve 900 line pair/mm, Germany) can be accomplished with our dual turret.

Statistical analysis
Statistical analyses were performed by the "Statistica for Windows, Release 7.0" and "Excel 2010" statistical package programs.

All parameters were tested for normal distribution and homogeneity of variance. The data was evenly distributed (Shapiro-Wilk test; $p > 0.05$), and was analyzed with a one-way factorial analysis of variance (one-way ANOVA). Depending on the homogeneity of variances, an LSD *post-hoc* test was applied for assessment of intergroup differences. Data are given as Mean±SEM, a Min–Max.

Differences were accepted as significant at the 95% level of confidence ($p<0.05$).

Pearson's correlation was performed to estimate the correlation of morphological measurements and hematological parameters and its strength.

The weight-length relationships of *C. gibelio* specimens from each site were performed by linear regression analysis.

The degree of informativeness and participation of each of the haematological parameters in differentiating the specimens of *C. gibelio* from the populations in the three compared sites were assessed by a Principal Component Analysis - PCA).

The relationship between the set of studied hematological parameters of *C. gibelio* specimens from all three sites and the parameters of the environment was assessed with the standard discriminant analysis. To constitute discrimination, parameters with factor weights > 0.7 were used. Discrimination between the specimens was performed on the basis of the extent and nature of anthropogenic pollution in the sites. The differences in Mahalanobis distance measurement are graphically presented through cluster analysis.

Table 2. *Results from the comparison of the morphological [(SL: standard length (cm); BW: body weight (g); FCF: Fulton condition factor)] and hematological parameters [(RBC: erythrocyte count (x 10^{12}/L); WBC: leukocyte count (N/mm^{3}); Hb: hemoglobin concentration (g/dl); PCV: packed cell volume (L/L); MCH: mean corpuscular hemoglobin (pg); MCHC: mean corpuscular hemoglobin concentration (g/L); MCV: mean corpuscular volume (fl)] in the individuals of Carassius gibelio from the sites investigated in*

Parameters	**Sites**			One-way ANOVA, post-hoc LSD-test	
	River Sazliyka below the village of Rakitnitsa. **Site 1** (n=30)	River Sazliyka below Radnevo. **Site 2** (n=30)	River Topolnitsa below the village of Poibrene. **Site 3** (n=30)	**F**	**Comparisons**
	Means ± standard errors of means Minimum–Maximum				
Morphological parameters					
SL	18.23±0.21 (16.80–22.50)	18.15±0.24 (16.30–21.30)	18.23±0.19 (16.30–19.80)	0.048	1/2ns, 1/3ns, 2/3ns
BW	131.25±2.66 (115.20–177.30)	126.24±4.45 (88.70–162.30)	115.42±3.42 (84.20–148.30)	5.089	1/2ns, 1/3**, 2/3*
FCF	2.176±0.04 (1.416–2.619)	2.095±0.03 (1.679–2.451)	1.892±0.02 (1.681–1.998)	21.261	1/2ns, 1/3***, 2/3***
Hematological parameters					
RBC	1.126±0.427 (0.870–1.700)	1.305±0.495 (0.950–1.800)	1.143±0.283 (0.850–1.500)	5.600	1/2**, 1/3*, 2/3**
WBC	2093±51.625 (1700–2600)	2816±65.929 (2200–3600)	1613±38.873 (900–1200)	129.158	1/2***, 1/3***, 2/3***
Hb	8.42±2.284 (6.91–12.84)	11.74±2.221 (9.52–14.08)	5.13±2.475 (3.73–7.74)	201.223	1/2***, 1/3***, 2/3***
PCV	0.24±0.007 (0.18–0.32)	0.29±0.005 (0.24–0.35)	0.22±0.004 (0.19–0.30)	43.024	1/2***, 1/3ns, 2/3***
MCH	78.57±3.008 (46.61–120.77)	94.77±4.119 (61.38–135.54)	45.35±2.450 (29.22–85.67)	59.431	1/2***, 1/3***, 2/3***
MCHC	356.31±11.605 (222.17–476.57)	396.43±11.151 (299.87–555.43)	222.30±12.599 (110.03–349.65)	59.709	1/2**, 1/3***, 2/3***
MCV	224.28±10.639 (147.33–352.78)	245.56±12.188 (157.35–391.67)	198.17±5.011 (133.67–247.72)	5.892	1/2ns, 1/3ns, 2/3***
Differential leukocyte count (N/100 WBC)					
Neutrophils	5.86±0.243 (4.00–9.00)	10.96±0.344 (8.00–15.00)	15.63±0.378 (12.00–19.00)	222.911	1/2***, 1/3***, 2/3***
Eosinophils	0.43±0.114 (0–2.00)	1.06±0.178 (0–3.00)	4.16±0.254 (2.00–7.00)	109.201	1/2*, 1/3***, 2/3***
Monocytes	2.23±0.156 (1.00–4.00)	3.70±0.198 (2.00–6.00)	6.83±0.325 (4.00–11.00)	97.482	1/2**, 1/3***, 2/3***
Lymphocytes	91.13±0.370 (83.00–93.00)	84.30±0.381 (81.00–88.00)	73.36±0.494 (69.00–79.00)	456.955	1/2***, 1/3***, 2/3***

Southern Bulgaria

Note: n – Number of individuals

* p < 0.05 (significant); ** p < 0.01 (more significant); *** p < 0.001 (most significant); ns - p > 0.05 (no significant)

RESULTS

The fish caught at site 2 (51) and site 3 (46) were only female. Among the fish caught at site 1 out of 59 altogether only 2 were male. A selection of 30 fish from each site, all being of the same age, (3+) and the same sex (all female), were used in the statistical analyses.

Morphological parameters

Results from statistical one-way ANOVA analysis of the values of morphological measurements of *C. gibelio* individuals from three investigated sites in two rivers are presented in **Table 2.**

SL - Standard length: there are no statistically significant differences in the values of this parameter in the fish from the three sites.

The parameters BW - Body weight and FCF - Fulton condition factor have the lowest statistically significant values in fish from site 3 (heavy metal pollution). There is no difference when fish from site 1 (less disrupted group) and fish from site 2 (domestic sewage pollution) are being compared.

The weight-length relationship of *C. gibelio* individuals from the three sites compared are shown in **Figure 2**. The values of the coefficient of determination (R^2) for the fish from sites 2 and 3 are close but highly than those of the fish from site 1. The scaling coefficients of fish in two contaminated sites 2 (17.289) and 3 (17.057) are different from those in reference site 1 (10.421) indicating a different growth pattern between these two populations of fish living in rivers with anthropogenic pollution.

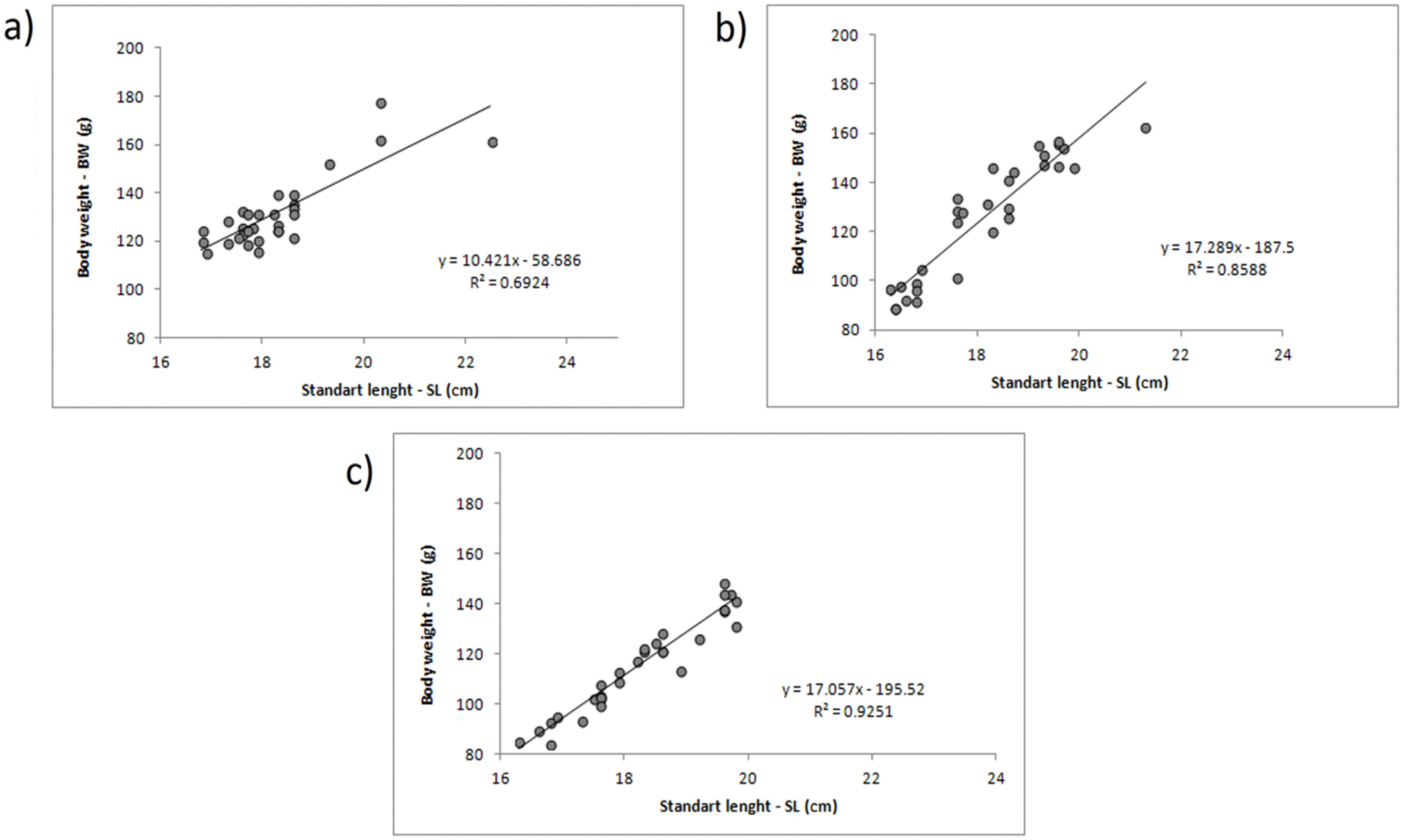

Figure 2. Regression analysis of relationships between body weight and standard length of *Carassius gibelio* from the sites investigated in Southern Bulgaria

Legend: **a**) site 1 – the river Sazliyka below the village of Rakitnitsa, **b**) site 2 – the river Sazliyka below Radnevo;, **c**) site 3 – the river Topolnitsa below the village of Poibrene

Hematological parameters

The results from statistical one-way ANOVA analysis of the values of hematological parameters of *C. gibelio* specimens from the three sites investigated in the two rivers are presented in **Table 2**.

RBC - Erythrocyte counts: the value of this parameter in the specimens from the less disrupted group (site 1) is statistically significant and lower than the values of this in the specimens from the two anthropogenically polluted sites. There is the statistically significant highest number of erythrocytes in the blood of the fish from site 2, which has domestic sewage pollution.

WBC - leukocyte count, HbC - hemoglobin concentration, MCH - mean corpuscular hemoglobin, and MCHC - mean corpuscular hemoglobin concentration: these parameters have the lowest statistically significant values in the blood of the fish from the site with heavy metal pollution (site 3) and the highest statistically significant value in the blood of the fish from site 2. There is no difference in the values of these parameters when comparing the specimens from site 1 and site 3.

MCV - mean corpuscular volume: the value of this parameter is the lowest in the blood of the fish from site 3 but the only statistically significant difference is observed comparing them with the fish from site 2. There is no difference among the specimens from sites 1 and 2.

Differential leukocyte count:
The numbers of neutrophils, eosinophils and monocytes in the blood of the fish from less disrupted group are statistically significant and lower than those in the blood of the fish in the two sites polluted by human activities. The number of neutrophils, eosinophils and monocytes in the blood of the fish from the site with heavy metal pollution (site 3) is the highest statistically significant.

Lymphocytes: this parameter has the highest statistically significant value in the blood of the fish from the control group and the lowest statistically significant value in the blood of the fish from the site with heavy metal pollution.

Correlation of body features and hematological parameters
It has been observed that for all fish in the three sites there was a significant correlation between body features and only one of the hematological parameters: packed cell volume **(Table 3).** For specimens from site 1 the PCV value was in negative correlation to BW ($p<0.05$). For specimens from site 3 the PCV value was in negative correlation to SL ($p<0.01$) and BW ($p<0.01$). However, for specimens from site 2, the PCV was in a positive correlation to the Fulton condition factor ($p<0.05$).

Table 3. *Person's coefficients between morphological and hematological parameters of Carassius gibelio individuals (n=30 from each sites) from the sites investigated in Southern Bulgaria*

Sites	Parameters	RBC	WBC	Hb	PCV	MCH	MCHC	MCV
1	SL	-0.283	-0.225	-0.061	-0.270	0.196	0.255	0.016
	BW	-0.342	-0.105	-0.056	-0.397*	0.255	0.344	-0.015
	FCF	0.144	0.355	0.047	-0.016	-0.101	-0.026	-0.096
2	SL	-0.172	-0.007	-0.248	-0.296	0.000	0.042	-0.009
	BW	-0.202	-0.111	-0.254	-0.152	0.052	-0.058	0.109
	FCF	-0.058	-0.249	0.050	0.377*	0.155	-0.220	0.295
3	SL	-0.178	0.056	0.107	-0.575**	0.196	0.258	-0.266
	BW	-0.240	0.101	0.124	-0.545**	0.209	0.241	-0.194
	FCF	-0.148	0.188	0.089	0.241	0.023	-0.075	0.281

Sites	Parameters	Neutrophils	Eosinophils	Monocytes	Lymphocytes
1	SL	0.282	-0.031	-0.131	-0.029
	BW	0.338	-0.216	-0.159	0.013
	FCF	-0.128	-0.135	0.051	0.050
2	SL	0.135	0.046	0.003	-0.123
	BW	0.093	-0.068	-0.049	-0.013
	FCF	-0.118	-0.279	-0.123	0.278
3	SL	-0.196	-0.001	0.041	0.124
	BW	-0.136	-0.065	0.030	0.118
	FCF	0.308	-0.302	-0.048	-0.049

Note: * Correlation is significant at the 0.05 level
** Correlation is significant at the 0.01 level

Multi-variational statistics – PCA
The sum of the first three variables (50.83%, 17.34% and 15.88%) includes 84.05% of the variation in specimens of *C. gibelio* from the three sites: eigenvalue was fixed ≥ 1.

The parameters Hb (0.862), Lymphocytes (0.849), MCH (0.837), MCHC (0.787), WBC (0.712), Eosinophils (-0.847), Monocytes (-0.772) and Neutrophils (-0.712) showed high degree of correlation in reference to the first axis (Factor 1). The parameters RBC (-0.950) and MCV (0.726), had a high correlation in reference to the second axis (Factor 2). The grouping of parameters in reference to the first two main axes is shown in **Figure 3.**

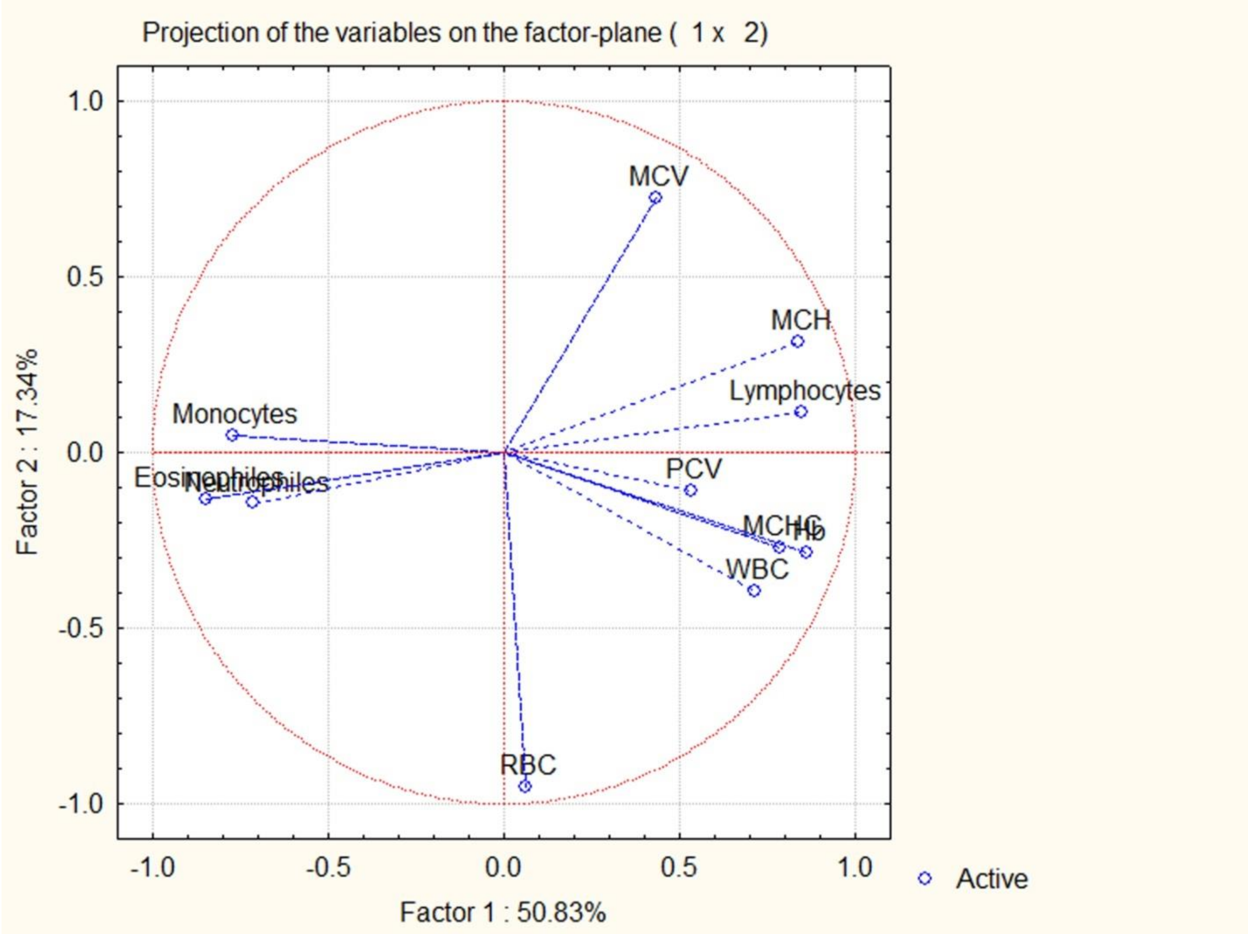

Figure 3. Graph of the correlations of 11 haematological parameters (factor weights) in the *Carassius gibelio* specimens from the sites with various degrees of anthropogenic pollution in Southern Bulgaria, to the first two main axes

Standard discriminant and cluster analysis

The discriminant function analysis (on the basis of the parameters RBC, Hb, MCH, MCHC, MCV, WBC, Lymphocytes, Eosinophils, Monocytes and Neutrophils) defined as statistically significant the difference between the compared groups of specimens *C. gibelio* from the three sites with various degrees of anthropogenic pollution in Southern Bulgaria. The parameters used were as follows: WBC (Wilks' Lambda = 0.012; F= 24.825; p = 0.001), Eosinophils (Wilks' Lambda = 0.008; F= 7.009; p = 0.001), Hb (Wilks' Lambda = 0.008; F= 6.780; p = 0.002), Neutrophils (Wilks' Lambda = 0.008; F= 6.260; p = 0.003) and MCHC (Wilks' Lambda = 0.008; F= 5.953; p = 0.004). The results of discriminative analysis distinguished most clearly the specimens of *C. gibelio* from site 3. According to the rate of increase in Mahalanobis distances, they were differentiated from the others as follows: 3/2 (101.437; F= 144.809; p < 0.00~1) and 3/1 (107.679; F= 136.415; p < 0.00~1). Mahalanobis distance is the smallest between the specimens of *C. gibelio* that live in conditions of domestic sewage pollution (site 2) and the individuals from the reference site 1 (28.995; F= 38.993; p < 0.00~1). Mahalanobis distances measured in the three sites are graphically presented in **Figure 4.** They show that *C. gibelio* specimens from site 3 can be clearly distinguished from the fish inhabiting sites 1 and 2.

The two canonical discriminant functions are statistically reliable for distinguishing the specimens of *C. gibelio* from the three sites, and the first function is responsible for 81.7% of the variance, while the second function has only 18.3% discriminative power. The analysis of the factor structure shows that the parameters RBC, Hb, MCH, MCHC, MCV, WBC and Lymphocytes positively correlate with the first canonical function. Simultaneously, the parameters Neutrophils, Eosinophils and Monocytes negatively correlate with the first canonical function. All hematological parameters, except Lymphocytes, have the biggest power, but they negatively correlate with the second canonical function **(Table 4).**

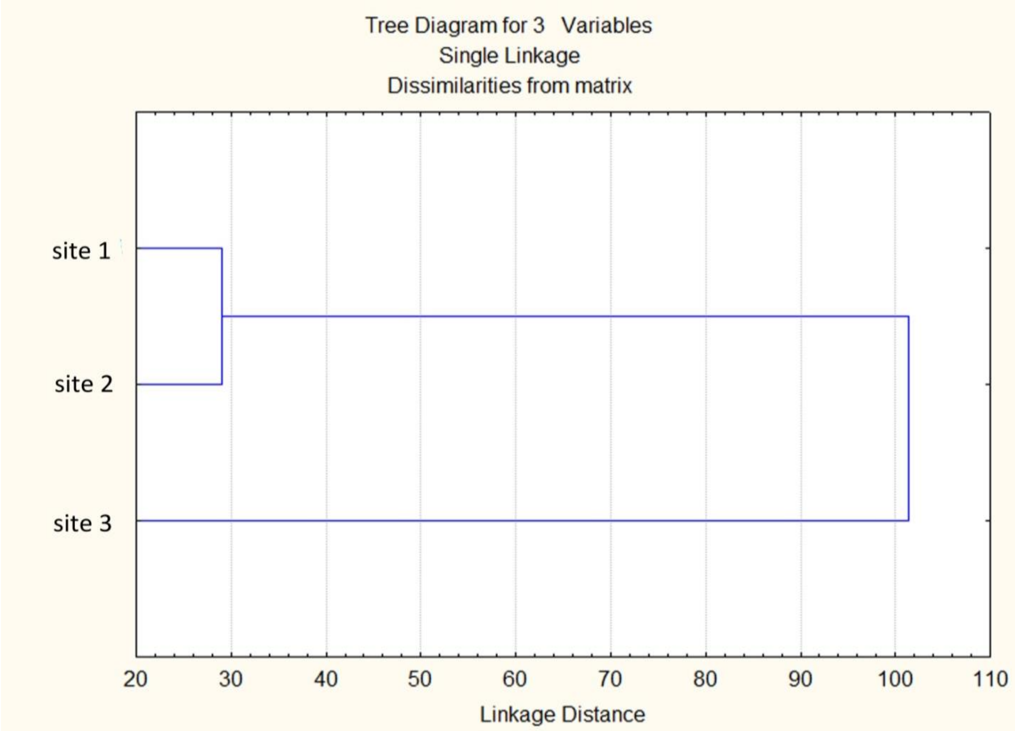

Figure 4. Cluster analysis plot – tree diagram for specimens of *Carassius gibelio* from the three sites investigated in Southern Bulgaria according to the received Mahalanobis distances

Legend: **a**) site 1 – the river Sazliyka below the village of Rakitnitsa, **b**) site 2 – the river Sazliyka below Radnevo, **c**) site 3 – the river Topolnitsa below the village of Poibrene

Table 4. *Factor structure matrix for fish hematological parameters and coefficients for canonical variables*

Variable	Root 1*	Root 2**	Raw Coefficients for Canonical Variables		Standardized Coefficients for Canonical Variables	
			Root 1*	Root 2**	Root 1*	Root 2**
RBC	0.029	-0.148	0.000	0.000	0.793	0.304
Hb	0.384	-0.513	-0.005	-0.088	-0.068	-1.127
MCH	0.231	-0.186	0.031	-0.029	0.548	-0.532
MCHC	0.239	-0.134	0.005	0.019	0.353	1.239
MCV	0.068	-0.079	0.007	0.013	0.399	0.728
WBC	0.284	-0.483	0.002	-0.002	0.471	-0.549
Neutrophils	-0.417	-0.498	-0.363	-0.226	-0.650	-0.405
Eosinophils	-0.332	-0.087	-0.539	-0.114	-0.565	-0.119
Monocytes	-0.304	-0.186	-0.261	-0.057	-0.340	-0.075
Lymphocytes	0.642	0.505	0.046	0.085	0.106	0.196
Constant	-	-	-10.646	-1.321	-	-
Eigenval	-	-	22.392	4.977	22.392	4.977
Cum.Prop	-	-	0.818	1.000	0.818	1.000

Note: * the first canonical function
** the second canonical function

The discriminatory coordinates for ten hematological parameters **(Figure 5)** show that the canonical discriminant functions clearly discriminate the specimens living in the two polluted sites (2 and 3) from those in the less disrupted group (1). Changes in the blood of fish from site 3 are clearly distinguished from both these inhabiting site 1 and these inhabiting site 2. At the same time, there is a partial overlap area in the values of hematological parameters in fish from the river with domestic sewage pollution (site 2) and those reported in the blood of fish from the less disrupted group (site 1). Basing on the results of discriminative analysis, we think that the changes in values of hematological parameters in specimens of *C. gibelio* from the two contaminated sites are due to the toxicants available in the middle. They are much more clearly expressed in the blood of fish from site 3. The value changes in the complex of hematological parameters have negative effect on the physical health of fish and it is strongly expressed in theTopolnitsa River that is contaminated with heavy metals.

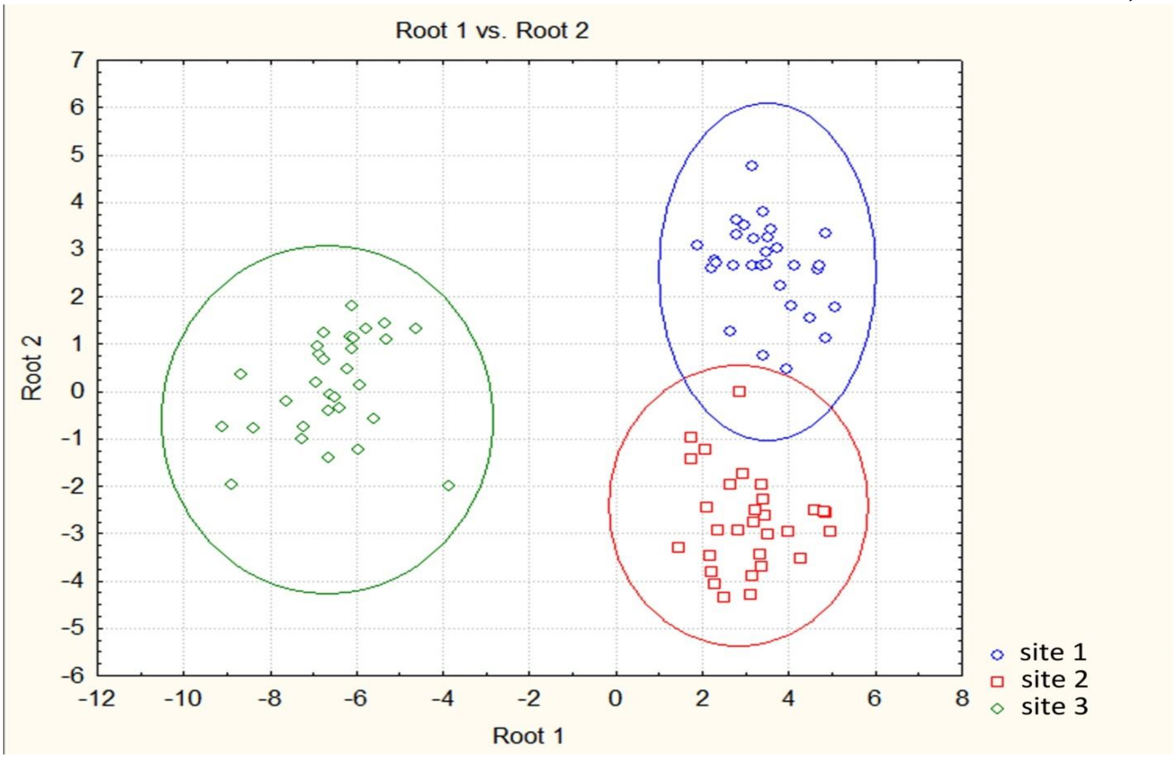

Figure 5. Plot (discriminatory coordinates for ten hematological parameters) of the importance of canonical discriminant function of the specimens of *Carassius gibelio* from the sites investigated in Southern Bulgaria (Root 1 - the first canonical function Root 2 **-** the second canonical function)

Legend: site 1 – the river Sazliyka below the village of Rakitnitsa, site 2 – the river Sazliyka below Radnevo, site 3 – the river Topolnitsa below the village of Poibrene

DISCUSSION

The length-weight relationship in fish is of great importance in fishery assessments. Studies on the conditions of fish showing that they are negatively influenced by environmental contaminants have been reported by Jenkins, 2004 (7) and Shobikhuliatul, 2013 (25).

In our study the analysis of morphological parameter values reflecting the general physical state of the specimens of *C. gibelio* inhabiting the three sites shows that the specimens from the river Topolnitsa have serious developmental problems.

The fact that the specimens are of the same age and there are no statistically significant differences in the SL parameter suggests that there are no growth disorders in the specimens from the three sites. At the same time the lowest statistically significant values of the BW parameter and the FCF parameter in the fish from site 3, which is polluted with heavy metals suggest serious developmental disorders. The causes can be found in the impoverished nutritional elements in the river as well as in the metabolism disorders triggered by the accumulation of heavy metals in vitally important organs such as muscles, liver and kidneys. The second hypothesis holds prospects for future studies and could be tested by analyses which were not the purpose of this study.

The analysis of the hematological parameter values obtained provides information about the hematological profiles of the *C. gibelio* specimens inhabiting the two anthropogenically polluted sites:

a) The changes in the parameters of erythrocytosis, leukocytosis and hyperchromia. Red cell indices (MCH, MCHC and MCV) in the blood of the specimens of *C. gibelio* from site 2 (domestic sewage pollution) have the highest statistically significant values. The changes in the differential leukocyte count are characterized by neutrophilia, eosinophilia, monocytosis and lymphocytopenia.

b) The changes in the blood of the specimens of *C. gibelio* from site 3 (heavy metal pollution) are characterized by erythrocytosis, leukocytopenia and hypochromia. The analysis of red cell indices (the values of MCH, MCHC and MCV are the lowest statistically significant) suggests that these specimens exhibit anemic changes of a microcytic and hypochromic type. The changes in the differential leukocyte count are characterized by neutrophilia, eosinophilia, monocytosis and lymphocytopenia. It is important to mention that the changes in the differential leukocyte count are more prominent in comparison with the respective changes in the specimens from site 2.

Since determination of the reference range of the hematological indicators in the poikilothermic animals is a difficult task, the comparison with the data from the literature is the only way for evaluating the fluctuations in their values and their dependence on the environmental factors and the anthropogenic pollutants.

Nikolov and Boyadzieva-Doichinova, 2010 (26) provides information about the RBC values in *C. gibelio* from an age-size group 9.5-12.0 cm. The average value they cite is 1.50±0.54 x 10^{12}/L. This value is higher than our result for the specimens in the control group (1.126±0.427 x 10^{12}/L) as well as the result for the specimens of the two polluted sites: 2 (1.305±0.495 x 10^{12}/L) and 3 (1.143±0.283 x 10^{12}/L). The reasons for this difference may be the small number (10) of the tested specimens, the lack of data about the animals' sex, the physico-chemical characteristics of the water and the higher altitude at which the water resource supplying the material used by the above mentioned authors originated.

In most fish, the kidney is the site for erythropoiesis (27). Previous research suggests that the number of erythrocytes in different bony fish in wild populations may vary according to the environment, the physiological activity or infections (28). The increased value of RBC indicates oxygen demand in the tropical region to meet the higher oxygen requirement at higher metabolic rates (29). The information about the effects of plant toxicants on erythropoiesis under *in-vitro* conditions is controversial – from its suppression (30) to lack of effect (7). According to Abdolazizi et al., 2011 (31) the higher concentration of some anesthetics such as clove oil cause a surge in the RBSs in the blood of *C. auratus* (Linnaeus, 1758), while other anesthetics such as Propofol have the opposite effect on *Coregonus lavaretus* Linnaeus, 1758 (32).

Research has shown that the most common cause of erythrocytosis in hydrobionts (fish and amphibians) inhabiting continuously places with anthropogenic pollution is hypoxia. Fish try to maintain tissue oxygen levels by increasing ventilation, altering blood flow patterns, and increasing red blood cell (RBC) counts and hemoglobin (Hb) concentration (33). Initial increases in RBC numbers are related to spleen contraction, but over the long term, RBC increases are related to increases in the glycoprotein hormone, erythropoietin EPO (34).

The increase in the number of erythrocytes, most often accompanied by hyperchromia, causes an increase of oxygen in the blood (35-36). These authors consider the stimulation of erythropoiesis a compensatory reaction to the long-term effect of the toxicants and partly to adaptation. Considering the examples provided it could be claimed that the valid increase of RBC cells in the blood of *C. gibelio* specimens from the two anthropogenically polluted sites increases their chances of survival in an environment with toxicants.

The values of the other two parameters associated with oxygen transport (Hb and PCV) in specimens of *C. gibelio* from the control group (8.42±2.284 g/dl and 0.24±0.007 L/L) which have been obtained in our study and those of site 2 (11.74±2.221 g/dl; 0.29±0.005 L/L) respectively, are higher that those reported by Nikolov and Boyadzieva-Doichinova, 2010 (26), 5.65±0.420 g/dl and 0.22±0.01 L/L respectively. The values of the two parameters for specimens from site 3 (5.13±2.475 g/dl; 0.22±0.004 L/L), however, are similar to those of the above mentioned authors. There have been very few studies on the altered values of Hb and PVC in fish populations inhabiting anthropogenically polluted water recourses. Studies conducted in southern Bulgaria (8) on the blood of three species of carp - *Alburnus alburnus* (Linnaeus, 1758), *Scardinus erythrophtalmus* (Linnaeus, 1758) and *Perca fluviatilis* (Linnaeus, 1758) inhabiting the Studen Kladenets Reservoir, which is polluted with heavy metals, show lower values of HB and PCV (fluctuating in spring and summer) than the values of specimen inhabiting the relatively clean Kardzhali reservoir.

Hemoglobin content of erythrocytes was associated with the volume and the development of RBCs. The effect of age on hemoglobin was determined to be insignificant. According to Hrubec et al., 2001 (37) levels of hemoglobin increased with increasing age. Environmental factors and genetic factors could have affected the development of erythrocytes (38). The fluctuation of MCH and MCHC could be due to the change of the hemoglobin concentration of RBCs in infected fishes (39). According to Kumar et al., 2013 (40) the decreased MCV can be the sign for a defect in the maturation of erythrocytes.

The high values of Hb and PCV as well as those of the red cell indices (MCV, MCH and

MCHC) in specimens of *C. gibelio* from site 2 are also a sign of pulling in the resources of the immune system and the blood circulation system to optimize the body functions in relation to the characteristics of the environment. The blood reactions in the specimens of *C. gibelio* from site 3 show disorders in the mechanisms of hemoglobin synthesis. This can explain the low values of Hb and red cell indices in this group. The causes of such disorders (anemia of microcytic and hypochromic type) can be found in the accumulation of heavy metals in such vital organs as the liver and spleen, which triggers deficiencies in erythrocyte growth or dysfunctions affecting the hemoglobin biosynthetic chains. This position is also supported by the results in the work of Kumar et. al., 2013 (40). However, there is the opposite claim that fish with low hemoglobin concentration preserve their vitality more successfully in an anthropogenically transformed environment (41). Such a position may be reasonable at low toxicant concentration or when there is a short-term activity affecting the hydrobionts. When there is a long-term existence in a toxic environment and the blood of the fish exhibits symptoms of anemia we believe that our claim is adequate.

Stress is thought to be responsible for leukopenia in fish. Environmental stressors such as toxicants are known to cause elevated plasma cortisol probably because corticosteroids depress inflammatory response, mobilization of leucocytes and phagocytosis (42). Toxicants may have impaired leucopoiesis leading to reduced WBCs (leukopenia) and possibly suppressing the immune (defense) system of the fish (43). There are studies which report the decrease of the number of WBCs in Teleostei fish under the effect of some anesthetics, such as clove oil (31), propofol (32) and pesticides (4).

The alteration of the differential leukocyte count is a reliable criterion for the evaluation of the fish organism when they inhabit anthropogenically polluted places. It is well established that under the influence of various toxicants the immune functions of fish's blood are reduced compared to those who live in relatively clean waters (41). According to Talkina et al., 2004 (44) the accumulation of mercury (Hg) triggers lymphocytopenia, monocytosis and neutrophilia in the blood of *Rutilus rutilus* (Linnaeus, 1758). In *Oreochromis mossambicus* (Peters, 1852) the accumulation of cadmium (Cd) leads to the destruction of mielocytites, decrease of lymphocytes and increase of the proportion of the cells with phagocytosis capabilities (45). The results in previous studies examining the alterations of differential leukocyte count in fish inhabiting anthropogenically polluted waters point out to leukocytosis regardless of the nature of the toxicants. Leukocytosis is most often accompanied by neutrophilia, while all the other parameters vary significantly: they can be lymphocytosis or lymphocytopenia, monocytosis or monocytopenia, eosinophilia, or the count of eosinophils stays the same (10, 46).

Neutrophils are active ferments and have phagocytotic functions. Neutrophil leukocytosis in the blood of fish and amphibians appears most often in infections and intoxications (35-36, 46, 47).

The results obtained in our study regarding the values of WBC counts and changes of differential leukocyte count support the claim that in the specimens of *C. gibelio* from site 2 there are processes of mobilization of the organism's immune system through stimulating the phagocytotic processes while in the fish from site 3 there are symptoms indicating disorders in the functions of blood organs and metabolism organs (spleen, kidneys and liver). These claims are supported by the results reported in the works of Brozio and Litzbarski, 1977 (46), Lai et al., 2006 (34), Shobikhuliatul, 2013 (25) and Zhelev et al., 2014b (48). The increased percentage of neutrophils and monocytes in the blood of the fish from the site polluted with heavy metals shows active neutralization of the products of tissue disintegration, and a weakening of the organism as a result of infections and parasite invasions (high count of eosinophils). An SA similar reaction of the immune system is described in the Indian major carp *Labeo rohita* Hamilton, 1822 (49) and in *Neogobius melanostomus* Pallas, 1814 (10).

CONCLUSIONS

- The lowest values of morphological parameters BW and FCF in the specimens of *C. gibelio*, which inhabit the site with heavy metal pollution, are indicators of disorders in growth processes and retarded physical development.
- The hematological profiles of specimens of *C. gibelio* from the river site with domestic sewage pollution: erythrocytosis, leukocytosis and hyperchromia, differential leukocyte count: neutrophilia, eosinophilia, monocytosis and lymphocytopenia, as well as the highest values of red cell indices (MCH, MCHC and MCV).
- The hematological profiles of specimens of *C. gibelio* from the river site with heavy metal

pollution: erythrocytosis, leukocytopenia and hypochromia, differential leukocyte count: neutrophilia, eosinophilia, monocytosis and lymphocytopenia, as well as the lowest values of red cell indices (MCH, MCHC and MCV) are changes that indicate anemic of microcytic and hypochromic type.

• Hematological parameters in *C. gibelio* found by us confirm other researchers' viewpoint that these parameters can be used as biomarkers in the system of ecological monitoring, independently alongside with pysico-chemical analyses. The alterations of hematological parameters may provide an early warning signals for the determination of toxic effects of chemicals on healthy of fish and to ascertain water quality of sites.

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