ASSESSMENT OF ECOLOGICAL STATUS AND PRELIMINARY RESULTS ON REFERENCE CONDITIONS IN ALPINE GLACIAL LAKES (BULGARIA) – A CONTRIBUTION TO THE IMPLEMENTATION OF THE WATER FRAMEWORK DIRECTIVE

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ABSTRACT
The 'normative definitions' of ecological water quality classes given by the Water Framework Directive (WFD) are narrative descriptions of the conditions present in water bodies of different qualities relative to reference conditions found in unimpacted sites. Defining the type-specific reference conditions for water bodies and finding biological metrics that sensitively reflect only the anthropogenic deviations from those conditions are the biggest challenges that the ecological status assessment faces. This study aimed at phytoplankton, macrophyte, macro-invertebrates and fish-based assessment in two high mountain glacial lakes and establishing preliminary reference conditions. The lakes are located in Rila and Pirin Mountains, Southwest Bulgaria. Chernoto Lake is ultra-oligotrophic and has abundant submerged macrophytes; Bezbog Lake is also oligotrophic with submerged macrophytes, both with macrozoobenthic communities typical for oligosaprobic conditions. Sampling was carried out from July to September in 2009 and 2011. The biological and water quality parameters in the lakes are discussed. Indices elaborated in a European context (Algae Group Index, Reference Index, adapted biotic index, Pantle & Buck's saprobic index and German trophic index PETI) were tested on the collected data set. The type-specific taxa for the studied lake type were preliminarily determined.

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Introduction
For correct evaluation and characterisation of a lake ecosystem it is considered essential to adopt an integrated approach that takes into account physical, chemical and biological metrics (phytoplankton, macrophytes, bottom macro-invertebrates and fish). It is recommended that the above biological indicators and, additionally, a range of supporting hydro-morphological and physico-chemical elements should form the core of any monitoring program on lakes (38).

The algal flora of 33 glacial lakes in Bulgaria, not including Chernoto and Bezbog, was studied (30). Following a mainly taxonomical and floristic publication on phytoplankton (37), phytoplankton studies in glacial lakes have proceeded with occasional data for abundance and vertical distribution at the Seven Rila Lakes (3, 4, 5, 22). An assessment of 78 lakes based on aquatic macrophytes in Bulgaria was carried out in 2009 (15). Determinations of aquatic oligochete fauna found in glacial lakes from Rila (34) and Pirin Mountains (35), including Bezbog and Chernoto lakes, were made from the collections of Prof. Valkanov, which were gathered during his expeditions between 1932 and 1968, devoted to studying life in high-mountain lakes.

Fragmentary information about the fish species inhabiting both the Rila and Pirin Mountains can be found in different sources (11, 12, 18, 21, 26) summarized by Karapetkova et al. (16) and Beron et al. (2). Some background information about the fishes in both the "Rila" and "Pirin" National Parks is presented in their Management Plans (19, 20). However, specific data about the fish fauna of neither the Bezbog, nor the Chernoto lakes were published.

Recently, a study of the main phytoplankton, macrophyte and zoobenthic characteristics of 80 lakes and reservoirs in Bulgaria was implemented by Cheshmedjiev et al. (7). Detailed integrated field investigations of glacial lakes in Bulgaria have not been carried out, although these ecosystems have environmental importance and sensitivity. Chernoto and Bezbog lakes have been influenced by many factors, such as increasing human pressure and trends in urban touristic development around the lakes. The present study aimed at (i) assessment of the ecological status of two selected lakes; (ii) establishing preliminary reference conditions and type-specific taxa at glacial lakes. Additionally, environmental variables were assessed to exclude possible eutrophication.

Materials and Methods
Spatial approach (selection of existing lakes with no or minor human impact) was applied to set the preliminary reference conditions following the guidance document (the REFCOND

guidance) on establishing reference conditions and ecological status for inland surface waters (13).

The sampling points at both lakes (**Fig. 1**) were selected with a view to be representative for the existing habitat diversity and homogeneity. Biological quality elements (BQEs) together with physico-chemical parameters of lake water were monitored in the period from July to September in 2009 and 2011.

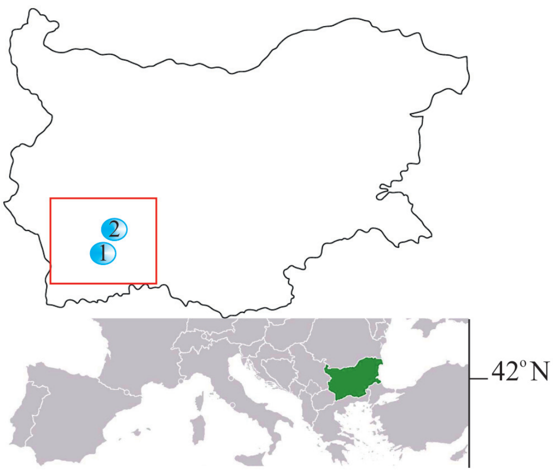

Fig. 1. Location of the studied lakes: No. 1 – Bezbog; No. 2 – Chernoto Lake.

Water quality parameters (temperature, pH, conductivity, transparency, dissolved oxigen and oxygen saturation) of lake water were measured *in situ*. Additionally, NH_4-N, NO_2-N, NO_3-N, PO_4-P, total nitrogen and phosphorus, COD, and BOD_5 were analyzed just after sampling on spectrophotometer NOVA 60 (MERCK) following adopted standards: ammonium nitrogen – ISO 7150/1, nitrite and nitrate nitrogen – EN 26777 and ISO 7890-1, total nitrogen (TN) – EN ISO 11905-1, phosphate phosphorus – EN ISO 6878, total phosphorus (TP) – EN ISO 6878, biochemical oxygen demand – EN 1899-1,2, and chemical oxygen demand – ISO 15705.

Phytoplankton seasonal (summer and autumn) observations were made during the second year. Discrete sampling from the deepest lake zone was carried out with a Ruttner batometer in 5 fixed median horizons: 0 m (surface area), 3.0 m, 7.0 m, 10 m, and 15 m (bottom layer). The general taxonomic scheme of Van den Hoek et al. (36) for phytoplankton was followed. At least 100 individuals were registered for the most numerous species per sample (23). The biovolume was determined following geometric form formulas (24), and was transformed into biomass according to Wetzel and Likens (39). The ecological status assessment was based on 4 main metrics (total phytoplankton biovolume, Algae Group Index, transparency according to Secchi, chlorophyll *a*) and 3 additional ones (% Cyanobacteria, presence of "bloom" and toxic species). Chlorophyll *a* concentration was determined spectrophotometrically, after extraction in 90 % ethyl alcohol, following a standard method – ISO 10260:1992. The intensity of the phytoplankton "bloom" was assessed on the basis of total biovolume on a 5-degree scale: I degree $\leq$ 2.5 $mm^3 \cdot L^{-1}$; II degree $\approx$ (2.5–10) $mm^3 \cdot L^{-1}$; III degree $\approx$ (10–500) $mm^3 \cdot L^{-1}$; IV degree $\approx$ (500–5000) $mm^3 \cdot L^{-1}$; V degree ("hyperbloom") $>$ 5000 $mm^3 \cdot L^{-1}$. In calculating % Cyanobacteria, some species/genera for oligotrophic waters were excluded, focusing on toxic species and eutrophic indicators (following WFD Intercalibration technical report, Part 2 – Lakes, Section 3 – Phytoplankton biomass metrics Annexes). The Algae Group Index (AGI, Catálan Index) was calculated for each sample (6). The interpretation of phytoplankton data based on the above metrics followed the classification system for the oligotrophic lake type in Bulgaria (7).

The taxonomic composition and abundance of aquatic macrophytes were recorded as biological metrics in the normative definitions of the ecological status classification in the WFD, Annex 1.2 (14). All submerged, floating-leafed, helophyte and amphiphyte species (Charophytes, Bryophytes and Tracheophytes) were recorded. Sampling was carried out following the recommendations of Schaumburg et al. (28). The taxonomy of vascular plants followed Flora Europaea (31, 32). The abundance of each species was noted on a five-degree scale (1 = very rare, 2 = infrequent, 3 = common, 4 = frequent, 5 = abundant, predominant) according to Kohler (17). Calculated Reference Index (1, 28) was transformed in EQR, where the value of "1" reflects the best possible ecological status.

An adapted version of the multi-habitat sampling method (8), applying different techniques according to EN 27828:1994 and EN ISO 9391:1995 was used for benthic macroinvertebrate fauna sampling. The assessment was based on an adapted version of the Irish Q-scheme (9, 10), Pantle and Buck's saprobic index (25) and Feeding type index (29).

Data about the ichthyofauna in the two target lakes and information about fish stocking were obtained by interviews with park staff and fishermen.

Results and Discussion

Reference conditions and respectively high ecological status based on water quality parameters and all BQEs were assessed at both lakes (**Table 1**). A slight deviation from reference conditions was illustrated by AGI during 2011. The transparency was not representatively evaluated due to high transparency – over maximum lake depth, especially at the ultra-oligotrophic Chernoto Lake, with biomass about 0. Chlorophyta*,* Chrysophyta*,* Bacillariophyta and Desmidiales were represented with the highest number of speices in Chernoto Lake; Desmidiales, Bacillariophyta and Cyanoprokaryota in Bezbog Lake (**Table 2**). Pyrrophyta, Chlorophyta, and Cryptophyta dominated at Chernoto Lake in respect to biovolume; Pyrrophyta*,* Bacillariophyta and Cryptophyta in Bezbog Lake*.* These results corroborated previous data for alpine lake biomass (3).

Among the general species richness at both studied lakes, the percent of Desmids and Diatoms was considerable and confirmed previous reported data (3, 37). Vodenicharov and Vodenicharov (37) assumed increasing of Chlorococcales as

TABLE 1

Morphometric characteristics, water and biological quality parameters in lakes

Lake	**Bezbog**			**Chernoto**		
Lake features						
Altitude, a.s.l. (m)	2240			2302		
Latitude	41 43 59.6			42 07 37.8		
Longitude	23 31 24.3			23 27 46.0		
Maximum depth (m)	7			15.5		
Water quality indicators	2009	2011		2009	2011	
T, °C	3	7.1		10.4	16.2	
Dissolved oxigen ($mg \cdot L^{-1}$)	7.9	9.3		7.9	8.2	
Oxigen saturation (%)	76	126		93	111	
pH	7.8	7.3		7.5	7.8	
Conductivity ($\mu S \cdot cm^{-1}$)	16	11.5		9.7	9.4	
SD (m)	>7	>7		>15.5	>15.5	
NH_4-N ($mg \cdot L^{-1}$)	<0.01	<0.01		0.01	<0.01	
NO_2-N ($mg \cdot L^{-1}$)	0	0		<0.002	<0.002	
NO_3-N ($mg \cdot L^{-1}$)	<0.2	<0.2		<0.2	<0.2	
TN ($mg \cdot L^{-1}$)	<0.5	<0.5		<0.5	<0.5	
PO_4-P ($mg \cdot L^{-1}$)	<0.01	<0.01		<0.01	<0.01	
TP ($mg \cdot L^{-1}$)	<0.01	<0.01		<0.01	<0.01	
COD ($mg \cdot L^{-1}$)	6.8	5.6		<4	<4	
BOD5 ($mg \cdot L^{-1}$)	<1	<1		<1	<1	
Biological Quality Element Metrics	2009	2011		2009	2011	
		Aug	Oct		Aug	Oct
AGI	0.48	0.92	0.99	0.55	0.99	0.99
Total biovolume ($mm^3 \cdot L^{-1}$)	0.95	0.43	0.17	0.57	0.02	0.04
Chl A ($\mu g \cdot L^{-1}$)	<0.2	< 1	< 1	<0.2	< 1	< 1
% Cyanobacteria	0.74	0	0	0	0	0
Blooms	0	0	0	0	0	0
RI	100	100		100	100	
Macrophytes - EQR	1	1		1	1	
MZB - Total quantity (m^2)	132	436		154	246	
% Oligochaeta	24	0		12	0	
Biotic index	n.a.	3.5		n.a.	4.5	
Potamal trophic index (PETI)	n.a	0.99		n.a.	0.83	

n.a.: not analyzed

TABLE 2

Relative abundance of phytoplankton based on taxonomic groups

Taxa	2009		Aug 2011		Oct 2011	
	Species number %	Biovolume %	Species number %	Biovolume %	Species number %	Biovolume %
	Bezbog Lake					
Cyanoprokaryota	9.1	0.7	15.2	0.3	25.7	0.5
Chlorophyta	18.2	6.9	3.0	0.3	5.7	0.3
Desmidiales	25.0	0.5	18.2	0.4	2.9	0.2
Chrysophyta	11.4	4.0	6.1	10.0	20.0	10.2
Bacillariophyta	29.5	9.5	51.4	56.3	31.4	11.8
Pyrrophyta	2.3	74.2	6.1	32.7	5.7	17.0
Cryptophyta	4.5	4.2	0.0	0.0	8.6	60.0
Total species number	44		33		35	
	Chernoto Lake					
Cyanoprokaryota	7.1	0.2	15.2	0.2	6.2	0.3
Chlorophyta	25.0	6.0	30.3	31.6	18.8	8.3
Desmidiales	14.3	0.4	12.1	0.3	31.3	0.4
Chrysophyta	21.4	5.4	6.1	26.7	15.6	5.6
Bacillariophyta	14.3	0.4	24.2	0.8	12.5	0.4
Pyrrophyta	10.8	77.6	9.1	0.4	6.2	0.3
Cryptophyta	7.1	10.0	3.0	40.0	9.4	84.7
Total species number	28		33		32	

an indication of an eutrophication trend, thus we accepted the Desmids and Diatoms domination in the summer phytoplankton as an indicator of reference conditions.

The results showed deviation of AGI from the reference value in conditions of no clear biomass domination of Dinoflagellates. The specific species composition and percent biomass allocation among divisions in the suited ultra-oligotrophic lakes, defined AGI values around 1, i.e. good status. This, in particular, was due to the equal percent participation of Colonial Chrysophyte and Colonial Diatoms, and, respectively, Chrysophyte and Diatoms not colonial in the total biomass, which took part in the AGI calculation. Thus further data accumulation is recommended towards correction of class boundary between high and good status.

Macrophytes illustrated high status with maximum value of Reference Index (RI). Macrophyte populations typical for alpine lakes were registered; they were dominated by *Isoetes lacustris* (Bezbog), *Subularia aquatica* and *Sparganium angustifolium*. *Sparganium angustifolium* had mass development in both lakes, having more solid fruits and broader leaves in the community dominated by Quillwort.

The above hydro- and hygrophyte species accounted for 50 % of the total abundance at Bezbog Lake. Helophytes and bryophytes were represented by an approximately equal number of species and abundance (27 % and 25 %, respectively). The share of indicator species abundance was 50 % and a maximum depth of plant colonization of 4 m was recorded.

Subularia aquatica and *Sparganium angustifolium* accounted for almost 90 % of the total abundance observed at Chernoto Lake. Helophytes and bryophytes were scarcely represented. *The i*ndicator species abundance was above 90 % and the maximum depth of plant colonization was 1.2 m.

The macrozoobenthic communities gave similar results. Taxa typical for oligosaprobic conditions were registered in Chernoto Lake: *Ecdyonurus eperoides*, *Capnia* sp., *Phylopotamus* sp., *Odontocercum hellenicus*, *Plectrocnemia conspersa*. Reference conditions and high ecological status were assessed at Chernoto Lake, based on the applied metrics (% Oligochaeta and Biotic index). The assessed trophic index characterized the ecosystem as stable and unaffected.

The macroinvertebrates illustrated near referent conditions and good ecological status at Bezbog Lake during the first year of observation. The anthropogenic impact was considered to be reduced based on three indices assessed in 2011, which illustrated high status. Nevertheless, some taxa typical for impacted waters were still registered (two Dipterean larvae: *Chironomus riparius* and *Cryptochironomus defectus*).

Both Bezbog and Chernoto lakes have been repeatedly stocked over the years with Brown trout (*Salmo trutta fario*) and, probably accidentally, with the alien species Rainbow trout (*Oncorhynchus mykiss*), similar to many other alpine lakes in Bulgaria. Furthermore, the Chernoto Lake was stocked with Brook trout (*Salvelinus fontinalis*), which is also an alien species of North American origin. In Bezbog Lake numerous

TABLE 3

Alphabetic list of preliminary type-specific taxa in alpine glacial lakes

Scientific name	Scientific name
Phytoplankton	**Macrophytes**
Cyanoprokaryota (Blue-green Algae)	*Isoetes lacustris* L.
Aphanothece elabens (Bréb. in Men.) Elenk.	*Sparganium angustifolium* Michx.
Coelosphaerium subarcticum Kom. et Kom.-Legn.	*Subularia aquatica* L.
Chrysophyta (Golden Algae)	**Macroinvertebrates**
Chrysococcus minutus (Fritsch) Nygaard	**Bivalvia**
Mallomonas tonsurata Teil. var. *alpina* (Pasch. et Ruttn.) Krieg.	*Sphaerium* sp. Scopoli 1777
Bacillariophyta (Diatoms)	**Ephemeroptera**
Aulacoseira italica (Ehrenb.) Simonsen	*Ecdyonurus epeorides* Demoulin 1955
Gomphonema acuminatum Ehr.	**Plecoptera**
Diatoma mesodon (Ehr.) Kütz.	*Capnia* sp. Pictet 1841
Tabellaria fenestrata (Lyngb.) Kütz.	*Nemoura cinerea* (Retzius 1783)
Tabellaria flocculosa (Roth) Kütz.	**Gastropoda**
Chlorophyta (Green algae)	*Ancylus fluviatilis* O.F. Muller 1774
Chlamydomonas passiva Skuja	*Radix balthica* (Linnaeus 1758)
Oocystis apiculata W. West	**Megaloptera**
Radiococcus nimbatus (De-Wild.) Schmidle	*Sialis lutaria* (Linnaeus 1758)
Trochiscia aciculifera (Lag.) Hansg.	**Trichoptera**
Conjugatophyceae, Desmidiales (Desmids)	*Halesus digitatus* (von Paula Schrank 1781)
Euastrum ansatum Ralfs	*Limnephilus sp.* Leach 1815
Euastrum bidentatum Näg.	*Odontocerum hellenicus* Malicky 1972
Euastrum didelta Ralfs ex Ralfs	*Philopotamus* sp. Stephens 1829
Euastrum verrucosum Ehr. ex Ralfs	*Plectrocnemia conspersa* (Curtis 1834)
Micrasterias rotata (Greville) Ralfs ex Ralfs	**Diptera: Chironomidae**
Sphaerozosma vertebratum Brébisson ex Ralfs	**Diamesinae**
Spondylosium lundellii Borge	*Diamesa* sp. Meigen 1835
Staurodesmus confergens (Ehr.) Teil.	**Orthocladiinae**
Teilingia excavata (Ralfs) Bourr.	*Brillia* sp. Kieffer 1913
Xanthidium antilopaeum (Bréb.) Kütz.	*Cricotopus (Cricotopus) algarum* (Kieffer 1911) *Eukifferiella gracei* (Edwards 1929)
	Tvetenia calvescens (Edwards 1929)

populations of Minnow *Phoxinus phoxinus* were presented. Although the Minnow is a native species in the waters of Bulgarian alpine lakes, it is considered a non-native one, introduced by coincidence with the trout fry and/or as bait used by anglers. In both of the studied lakes angling (and incidental poaching) is practiced. Thus, both in Bezbog and Chernoto lakes the ichthyofauna is subjected to long-term human impact mainly through artificial stocking (including introduction of alien and non-native species) and, to a lesser extent, by fishing pressure. The available data do not allow defining even retrospectively a referent state of the ichthyofauna. Therefore the lakes are considered unrepresentative for fish-based assessment.

Based on species composition during the studied period, 23 phytoplankton, 3 aquatic macrophyte, 17 macroinvertebrate preliminary type-specific taxa were found (**Table 3**). *Isoetes lacustris* is among the most common species in European lakes and is classified as a reference species, which should have very low abundance or be completely absent in impacted lakes (27). Monodominant coenoses of quillwort are widely distributed in Pirin Mountain glacial lakes, with high (70 % to 80 %) projective cover of the above-ground parts (33).

Thus, although restricted to Pirin Mountain lakes distribution, *Isoetes lacustris* was listed as a type-specific taxa.

It has to be emphasized that the attempt to assess reference conditions, even those of the same lake type, needs continuous researches and the list of type-specific taxa needs further evaluation, since no two lakes are alike, and therefore, despite the similar basic conditions, no floras and faunas are alike.

Conclusions

The phytoplankton, aquatic macrophytes, macroinvertebrates and fish investigated together with the main environmental factors illustrated specific ulra-oligotrophic ecosystems with low phytoplankton quantity and scarce macroinvertebrate communities. Fish were found to be completely unrepresentative and were excluded from assessment of national type L1. Additional calibration of AGI is recommended towards specific phytoplankton communities which naturally showed higher index value, minimum biomass and rich species diversity. More data are necessary for class boundary validation of scale for glacial lakes based on phytoplankton.

The preliminary list of type-specific taxa for reference conditions as indicators for high ecological status in glacial alpine lakes was reported to indicate oligotrophic conditions. These taxa should be represented at least by 50 % in waters having a high ecological status.

The overall ecological status assessment demonstrated that both lakes were unaffected with undisturbed reference conditions.

The results obtained increase the knowledge about the glacial lake ecosystems and are basis for future researches. These preliminary reference conditions can be applied, through a comparison with observed BQEs at a site, in the assessments of ecological status. Additional biological quality elements (zooplankton and phytobenthos) should be considered for monitoring under the WFD in further assessment.

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