Presence and identification of *Legionella* and *Aeromonas* spp. in the Great Masurian Lakes system in the context of eutrophication

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ABSTRACT

Growing anthropopressure over the last several decades has resulted in rapid progressive eutrophication of the Great Masurian Lakes (GML) system located in northeastern Poland. In our studies, we investigated whether there is a relationship between the occurrence of pathogenic bacteria: *Legionella* spp. and *Aeromonas* spp., not explored so far in the waters of GML system, and the trophic status of the studied lakes. The GML system of glacial origin includes lakes connected by natural and artificial channels, and it extends from north to south for approximately 100 kilometers. Water samples were taken during the summer, subsequently spring and autumn seasons from 15 lakes in land-water ecotones. At all sampling sites, basic *in situ* measurements of physicochemical parameters were recorded. The amounts of chlorophyll *a*, nitrogen, phosphorus, dissolved organic carbon were also measured. The trophic state index (TSI) of the sampling sites was also estimated. The real-time PCR technique enabled the determination of the presence and abundance of *Legionella* spp. and *Aeromonas* spp. The results clearly showed that several environmental water quality parameters, associated with eutrophication, and among them: nitrogen, phosphorus, chlorophyll, ammonium concentration, conductivity, turbidity, water transparency, highly affected the presence and abundance of the detected pathogenic bacteria in the studied lakes. Special attention should be paid to the high impact of water eutrophication on the number of pathogenic microorganisms, which result both from human activities in lakes and climate change.

INTRODUCTION

The contamination of water bodies by water-borne pathogens and the human health safety related to the pathogen contamination are some of the major water quality concerns around the world (Pandey, 2014). According to The World Health Organization (WHO), approximately 3.4 million people, mainly children, die from water-related diseases every year (WHO, 2016). Water-borne diseases are a problem not only in developing countries (although they are prevalent) but also in well-developed countries (Arnone and Walling, 2007; Wacnik, 2009). Studies conducted by the U.S. Environmental Protection Agency revealed that pathogens are leading factors of water impairment (Pandey *et al*., 2014). In turn, all infectious diseases caused by bacterial pathogens are major causes of death throughout the world (Binder *et al*., 1999), which demonstrates the great danger posed by pathogenic microorganisms. Many infectious diseases are caused by anthropogenic disturbances to aquatic systems, including the overuse of water resources, climate change and animals and human pollution impact (Ahmed *et al.,* 2016; Cavicchioli *et al.,* 2019). To predict the survival and transport of pathogens at the watershed scale, several models were developed (Dorner *et al.,* 2006; Cho *et al.,* 2016). However, many pathogen transport models consider only temperature-induced mortality and growth, and they omit the interplay with other environmental water quality factors, such as biogen (nitrogen, phosphorus, carbon) concentration, organic matter content, and several physical and chemical properties of the aquatic environment. Greater amount of nutrients increases the amount of sediments, which are a reservoir and source of nutrients for pathogenic bacteria (Pandey *et al.*, 2014). The presence of microorganisms is also influenced by hydrometeorological changes such as heavy rainfalls and elevated temperatures which increase with climate change (Jung *et al.,* 2014). Rainfalls cause increased surface runoff, providing allochthonic microflora and enriching the aquatic environment with biogenic substances from the catchment area. That allows the development of both autochthonous and allochthonous microflora. Furthermore, several studies

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have shown consistent and significant association between heavy rainfall events and waterborne disease outbreaks (Jung *et al*., 2014; Levy *et al*., 2016).

In natural and pristine water ecosystems, phosphorous or nitrogen contents are the limiting factors for plankton biomass production. However, human activity has changed this significantly and accelerated the supply of nutrients; as a consequence, the rates and scales of eutrophication have also increased (Correll, 1999).

Great Masurian Lakes (GML) system consisting of the two hydrologically different parts separated by the line of the watershed placed between Kisajno lake and Niegocin lake (Fig. 1). Both parts of GML system differ distinctly in respect to their morphometry, the trophic status and the level of human impact (Siuda and Kiersztyn, 2014). Present trophic status of the GMLS was shaped by four basic factors: i) geographical location - its division into two separate parts carrying waters into two watersheds and evolving in a diverse way; ii) relatively low anthropogenic impact on catchment areas of northern lakes and strong anthropopressure exerted simultaneously on the catchment areas of southern lakes; iii) political economic and social changes in Poland in the years 1980-1990, which increased a tourism in the region; and iv) climate changes occurring in the last few decades, resulting in an increase in mean daily temperatures in winter, shorter ice cover, and also shortening of autumn and spring homothermic periods (Siuda *et al.,* 2020). Because the microbial contamination of water is often caused by discharge of polluted water from sewage treatment plants as well as from non-collective sewage systems, it is important to monitor microbiological hazards of natural waters. Microbiological and sanitary monitoring of water commonly rely up on simple and rapid indicators, such as fecal bacteria (*Escherichia coli* or *Enterococci*) (Jung *et al.,* 2014). So far, the presence of *Legionella* and *Aeromonas* spp. in the water reservoirs of the system of the Great Masurian Lakes has not been monitored.

A significant number of studies on microbial pathogen contamination of aquatic systems have been conducted at a laboratory-scale; however, to understand the transition and survival of pathogens in natural water environments, field-scale studies are needed (Pandey *et al*., 2014). One of the studied pathogens of our interest was *Legionella* genus*,* which covers 61 species, and among them, 22 species are responsible for human disease. *Legionella pneumophila* is responsible for the largest number of legionellosis (Lizana *et al*. 2017). Pathogenic strains of *Legionella* spp. are etiological factors of Legionnaires' disease – severe, life-threatening pneumonia – and a less-severe disease called Pontiac Fever. Contagion occurs by inhalation of *Legionella*-contaminated water aerosols. *Legionella* spp. upon transmission to human infect and replicate within alveolar macrophages (Pasqualina *et al*., 2017). The capacity to replicate in human macrophages is related with the innate ability of *Legionella* to replicate within various free-living protozoa. This ability is one of the strategies to survive in unfavorable conditions (Amaro *et al*., 2015). The host cells enable bacterial replication, and viable released bacteria are more virulent than *Legionella* spp. that bypassed intracellular multiplication (Richards *et al*., 2014; Correll, 1999). *Legionella* spp. are also able to be transformed into a viable but nonculturable forms. All these strategies allow *Legionella* to resist to biocide compounds and chlorination (Borella *et al*., 2005). Moreover, these bacteria are remarkably fastidious in axenic cultures which makes it difficult to detect using culture methods. Therefore, real-time PCR is an appropriate method for detecting lower levels of contamination, as well as nonculturable *Legionella* (Devos *et al*., 2005; Edagawa *et al*., 2015). *Legionella* are bacteria strictly associated with man-made water systems such as cooling towers, swimming pools, air conditioner systems, and plumbing systems. However, these microorganisms are numerous also in natural water reservoirs (Barna *et al*., 2015). Many studies have shown, that these bacteria multiply at temperature ranging from 20 to 45°C (Dimitriadi and Velonakis, 2014). However, *Legionella* spp. may adapt to aqueous environments even at low temperatures and are able to survive over a wide range of temperatures: from 0 to 63°C (Nguyen and Yu, 1991).

Aeromonas was the second genus of bacteria that we focused on in our studies. Today these bacteria are described as emerging pathogens (Batra *et al*., 2016). *Aeromonas* are strictly associated with the aquatic environment and were firstly described as pathogens of fishes and other cold-blooded animals. Currently these bacteria are also recognized as a human pathogen. The interest in this genus has increased over previous decades. This is due to widespread occurrence, the increasing antibiotic resistance and ability to survive under unfavorable environmental conditions (Janda and Abbot, 2010). *Aeromonas* spp. with *Aeromonas hydrophila* at the forefront, are associated with gastrointestinal, skin, soft tissue, respiratory and urinary tract infections in both immunocompetent and immunocompromised persons (Martino *et al.,* 2014). Because classic culture and biochemical methods for identifying *Aeromonas* spp. are multi-stage and difficult to interpret, there is need to use molecular methods for reliable identification of *Aeromonas* spp. (Beaz-Hidalgo *et al*., 2010; Králová *et al.,* 2016).

The goal of our research was to detect and quantify of *Legionella* spp. and *Aeromonas* spp. with particular emphasis on *Legionella pneumophila* and *Aeromonas hydrophila* species in lake water with different trophic status belonging to the GML system. We evaluated whether the eutrophication processes in the studied lakes influenced the frequency of occurrence of the studied

pathogenic strains of bacteria. The research is important in view of complementing the current state of knowledge concerning the relationship between environmental water quality properties and the presence frequency of *Aeromonas* and *Legionella* species. Therefore, in our report, we present the results of studies on the relationship between environmental conditions and the presence and abundance of pathogenic bacteria strictly associated with the limnological quality of the water environment – *Legionella* spp. and *Aeromonas* spp.

METHODS

Research area

Our research area comprises lakes connected by natural or artificial channels that constitute the Great Masurian Lakes (GML) system located in northeastern Poland, which extends for approximately 100 km from south to north. The GML system is located in two river basins. The watershed location is not strictly determined, but it varies between Kisajno Lake and Jagodne Lake. This contractual boundary divides the system into northern (lakes: Przystań, Mamry, Dargin, Kisajno) and southern parts (lakes: Niegocin, Boczne, Jagodne, Szymoneckie, Szymon, Tałtowisko, Tałty, Ryńskie, Mikołajskie, Bełdany, Śniardwy) (Fig. 1). The water reservoirs that constitute this exceptional waterway represent moraine and channel types of postglacial lakes. The part of Poland that comprises the GML system is called the Great Masurian Lake District (GMLD). This part of Poland is a unique area formed by ice sheets of the Pomeranian Phase of the Vistulian Glaciation in the late Pleistocene (Wacnik, 2009). The basic morphological parameters of the studied lakes are presented in Tab. 1. The GML District is characterized by the highest surface water content in Poland. The entire catchment area of the GML system encompasses approximately 3645 km^2, of which, the northern lakes cover 615 km^2 and the southern lakes cover 3030 km^2. This size difference is one reason for the greater exposure of southern lakes to eutrophication processes. The system is located in the same geographical area under similar geological conditions. The area surrounding the lakes is mainly represented by agriculture fields and forests (Chróst and Siuda 2006). Water from the northern lakes is drained by the Węgorapa River to the Pregoła River basin, and water from the southern lakes is drained by the Pisa and Narew Rivers towards the Wisła River basin.

The GMLD is of great importance for tourism and the economy and is intensively used for recreational purpose especially during the summer season (Siuda *et al*., 2019). The number of tourists in the Masurian region reaches about one million persons per year, with the highest peak from June to August (Kauppinen, 2013).

The sampling procedure was conducted by considering places within easy reach of humans, such as watering locations, ports or sites for water sport practice. The sampling sites were selected by the largest probability of both introduction of allochthonous microflora and human contact with contaminated water, and consequently, the greatest possibility of infection.

Tab. 1. Basic morphological data of the studied lakes, coordinates of sampling locations and mean trophic state index determined during particular research seasons.

Lake	Area (ha)	Max depth (m)	Mean depth (m)	Coordinates of sampling location	Mean TSI summer	Mean TSI spring	Mean TSI autumn
Przystań	115	22.8	13.4	54.207241, 21.657892	42.18±2.18	43.17±2.25	41.12±2.96
Mamry	2 504	43.8	10	54.157234, 21.723144	40.20±8.98	44.22±2.55	39.40±3.85
Dargin	3 030	37.6	11	54.145939, 21.731939	42.77±0.96	41.87±4.84	51.69±15.27
Kisajno	1 896	25	8.4	54.042000, 21.738594	52.59±2.99	43.8±1.50	49.98±6.45
Niegocin	2 600	39.7	9.9	54.009215, 21.738598	48.47±2.76	48.25±3.54	54.27±8.93
Boczne	183	25	8.4	53.967407, 21.758265	50.21±4.21	50.32±4.35	53.20±10.53
Jagodne	420	37.4	8.7	53.945283, 21.721688	58.53±2.98	57.95±2.95	57.27±4.69
Szymoneckie	523	28.5	8.7	53.918687, 21.697991	59.27±2.28	55.71±3.27	56.07±5.07
Szymon	154	2.9	1.1	53.891046, 21.633682	57.58±1.34	58.48±1.27	58.08±1.67
Tałtowisko	327	39.5	14	53.880376, 21.560412	56.42±3.00	56.97±4.14	52.73±5.38
Tałty	1 160	50.8	14	53.813409, 21.567998	59.33±3.83	55.33±2.72	53.10±5.99
Ryńskie	671	20.2	13.5	53.935961, 21.544402	59.91±1.21	58.83±4.92	54.50±4.91
Mikołajskie	498	25.9	11.2	53.801142, 21.570925	59.14±2.04	55.52±2.45	53.94±5.68
Bełdany	941	46	10	53.686344, 21.578000	60.54±1.23	58.76±2.09	56.39±3.51
Śniardwy	11 340	23.4	5.8	53.766621, 21.842127	42.07±3.82	42.17±1.81	50.53±4.67

Sampling and experimental procedure

Water samples were taken from the water column of 15 lakes in July 2016, and subsequently from the same sampling sites in May 2017 and in September 2017. Sampling sites were located within land-water ecotones (from 10 to 50 m distance from the lake shorelines). The sampling sites have been chosen in lake areas exposed to high human recreational activities (swimming areas, yachting ports, *etc.*). The samples were taken using sterile sampling bathymeter, that was used to take water samples

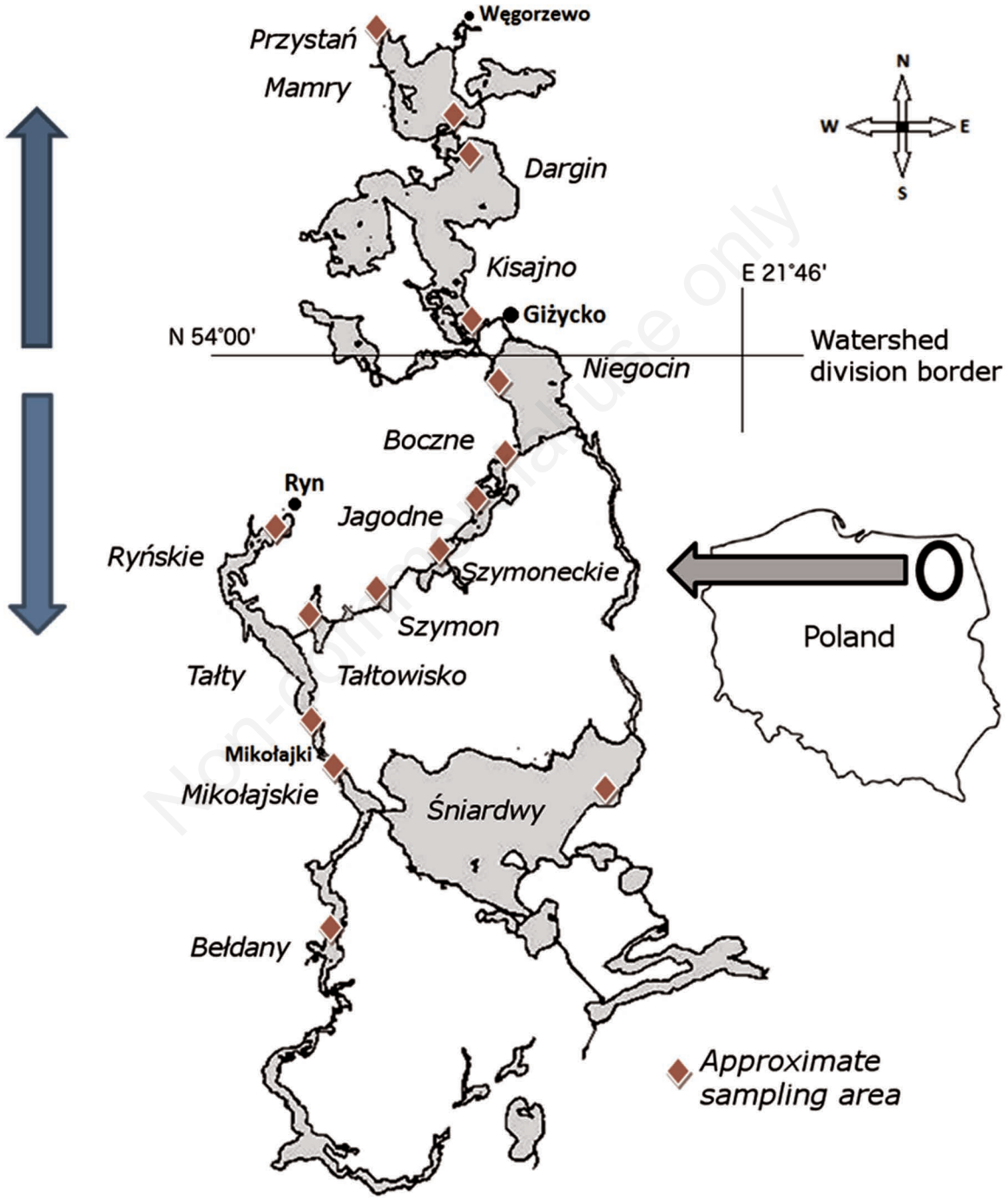

Fig. 1. Locations of sampling sites within the Great Masurian Lakes system. The blue arrows indicate the water flow direction. From the watershed division border to the north the northern lakes are located, from the watershed division border to the south there are southern lakes.

at a desired depth without the risk of mixing with water from other depths. We took the samples from randomly chosen points within a radius of 30 m from each of the main sampling sites. The sampling sites are shown in Fig. 1. From every sampling point equal volume of water was sampled from three depths: 1, 2 and 3 meters. All subsamples (about 1.67 L of every subsample) were mixed v/v to a volume of 5 liters and transported as soon as possible under cool temperature conditions to the laboratory where they were subjected to further analysis within 3-6 hours. Using a multiparametric probe YSI6600 (Yellow Spring, USA), the temperature, conductivity, oxygen concentration, pH and turbidity in the water column (from the water surface to bottom sediments) were measured *in situ*. The transparency of the water was also measured using a Secchi disk (SD) visibility survey.

Chlorophyll *a* (Chl a) content in all water samples was determined according to Arrar and Collins (1997). For this purpose, extraction of 10 mL water sample with 10 mL of 98% acetone and fluorescence measurements at 750 nm, with use of a TD-700 fluorometer were carried out. Dissolved organic carbon (DOC) concentrations in water samples, prefiltered through 0.2 µm pore-size polyethersulfone membrane filters (Merck Millipore, USA), were determined using a Shimadzu TOC 5050 carbon analyzer with a detection limit and accuracy of ±50 µg C L^{-1} (Chróst and Siuda, 2006). Phosphorus concentrations, fractions of total phosphorus (TP) and orthophosphates ($P\text{-}PO_4$) were determined spectrophotometrically according to Koroleff (1983a) using Shimadzu UV-VIS 1201 spectrophotometer. Total nitrogen (TN) was determined using commercially certified Merck-Millipore cell tests [Spectroquant Nitrogen (Total) Cell Test, 114537] according to the manufacturer's instructions using Merck Spectroquant Pharo 300 spectrophotometer. The ammonium concentrations in the water samples were assayed fluorometrically in a Shimadzu RF 1500 spectrofluorometer, according to Holmes *et al*. (1999).

Trophic state index

The trophic state index (TSI) at each sampling site was calculated based on chlorophyll *a* (Chl *a*), total phosphorus concentration (TP) and Secchi disc visibility (SD) according to Carlson (1977). To calculate the TSI, the following equations were used, respectively:

$$TSI\ (Chl\ a) = 9.81 \ln (Chl\ a \text{ in } \mu g\ L^{-1}) + 30.6;$$
$$TSI\ (TP) = 14.42 \ln (TP \text{ in } \mu g\ L^{-1}) + 4.15;$$
$$TSI\ (SD) = 60 - 14.41 \ln(SD \text{ in m}).$$

Subsequently, the TSI values calculated separately on the basis of the above, mentioned indicators were averaged, and the mean values of the trophic state of the sampling sites were determined. TSI values between 30 and 40 indicated oligotrophy, values between 40 and 50 indicated mesotrophy, values from 50 to 70 indicated eutrophy, and TSI values above 70 indicated hypereutrophy.

DNA extraction and amplification

A total of 150 mL of water from each sample was filtered through polycarbonate membranes with pore sizes of 0.2 µm (Nuclepore, Whatman, UK). The filters were placed in sterile 1.5 mL Eppendorf-type tubes and immediately frozen at -30°C until further DNA extraction. DNA extraction was done using the GeneMATRIX Soil DNA Purification Kit (EURx, Poland) according to the instruction manual supplied by the manufacturer with the modification that concerned the preparation of filters for DNA isolation. The filters were fragmented using sterile laboratory scissors in a bead beating tube containing beads and lysis solution. The aim was to lyse the microorganisms in the filters by a combination of heat, detergent and mechanical force against the beads. Specialized solution was added to precipitate humic substances that strongly inhibit downstream applications. Optimized buffer and ethanol provided selective conditions for DNA binding to the DNA binding spin-columns. Contaminants remaining on the resin are efficiently removed in two washing steps. High-quality DNA was then eluted in low salt buffer. Isolated total DNA was checked for quality and quantity by agarose electrophoresis and a Synergy H1 microplate reader (Gen5 software, BioTek, USA), respectively, and the samples were subsequently stored at -20°C prior to further analysis.

To determine the presence and number of microorganisms of the genus *Legionella* and the species *Legionella pneumophila*, the commercial and specific mericon Quant *Legionella* spp. Kit and mericon Quant *L. pneumophila* Kit (Qiagen, Germany) were applied. These kits are a ready-to-use systems for the detection of specific DNA fragments from *Legionella* spp. and *L. pneumophila* in water, food, animal feed and pharmaceutical products using real-time PCR. Using the above mentioned kits, real-time PCR detection and quantification were carried out according to the instructions provided by the manufacturer. The reaction mix per each sample contained 5 µL of *mericon* Assay inclusive Multiplex PCR Master Mix, HotStarTaq Plus DNA Polymerase and specific primers and probes. In case of samples which formed standard curves, the 5 µL of respective standard dilutions (from $1.25x10^1$ to $1.25x10^4$ cells per reaction) of prepared from purified *Legionella spp.* and *L. pneumophila* standard DNA were added. Additionally, Quantification control as positive control was applied with 5 µL of Quantification control with *Legionella* spp. and *L. pneumophila* DNA respectively. Moreover a negative control was applied. An

appropriate amount of DNA, according to manufacturer's instruction (50ng), in case of tested samples were added to the final reaction volume of 10 µL. The reaction runs for the quantification of *Legionella* spp. and *L. pneumophila* cells were as follows: polymerase activation for 5 min at 95°C and 40 cycles comprising denaturation for 15 sec at 95°C and annealing and plate read for 23 sec at 60°C and final extension for 10 sec at 72°C. The quantitative detection of *Aeromonas* spp. was based on the detection of the gene fragment encoding the conserved gyrase B subunit (*gyrB*) using primers sequences according to Khan *et al*. (2009) (forward primer: 5'-CTGAACCAGAACAAGACCCCG-3', reverse primer: 5'-ATGTTGTTGGTGAAGCAGTA-3'). The size of amplified fragment was 130 bp. Regarding the detection and quantification of *A. hydrophila* in the studied lake water samples, amplification were conducted using the specific primers for gene encoding the *Aeromonas hydrophila* adhesin (*ahaI*), according to Sebastião *et al*. (2018): forward primer 5'-GAGAAGGTGACCACCAAGAACA-3' and reverse primer 5'-GAGATGTCAGCCTTGTAGAGCT-3'. The length of *ahaI* fragment was 200 bp. In the case of *Aeromonas* spp. as well as *A. hydrophila* real-time PCR detection and determination were applied using the iTaq™ Universal SYBR®Green Supermix reaction mixture (Bio-Rad, USA). The reaction mixture per sample contained 5 µL of 1x concentrated iTaq™ Universal SYBR® Green Supermix, 0.5 µM of each primer, around 50 ng of DNA template and nuclease-free water to the final volume of 10 µL. All amplification reactions carried out in this study, both for *Legionella* and *Aeromonas*, were done in triplicates. A negative control was also applied. In *Aeromonas* spp. quantification analysis the standard curve was prepared using quantified genetic material isolated from sequenced total DNA of *Aeromonas* spp. (accession in Sequence Read Archive: PRJNA523334). *A. hydrophila* quantification was conducted with reference to standard curve prepared from genomic DNA of *A. hydrophila* ATCC 7966 (Minerva Biolabs, Germany). The numbers of *Aeromonas* and *Aeromonas hydrophila* were calculated based on the measured DNA concentration and the length of the genome sequence. Then a tenfold series of dilutions (ranging from 10^6–10^0 cells) were prepared. This was used to determine both the limit of detection of each assay and to calculate cells number. The minimal reaction efficiencies were of 90–100% and $0.997 < R2 < 0.999$. All amplification reactions were performed using a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, USA). The reaction run for the quantification of *Aeromonas* spp. and *A. hydrophila* was as follows: polymerase activation for 5 min at 95°C and 40 cycles comprising denaturation for 5 sec at 95°C and annealing, extension and plate read for 30 sec at 60°C. Finally, melt curve analysis was conducted over a temperature gradient from 65 to 95°C at 0.5°C increments at 5 sec per step. The real-time PCR results were analyzed automatically after entering the standard curve concentrations in the Bio-Rad CFX Maestro 1.1 software. Then the results were calculated into cells per milliliter and liter.

Statistical analysis

The analyses consisted of series of statistical test. Nonparametric Kruskal-Wallis test was used to check if the lakes were statistically different in terms of amounts of bacteria. To group the lakes according to the *Legionella* and *Aeromonas* occurrence profile and physicochemical parameters, two Bray-Curtis based non-metric multidimensional scaling (NMDS) analyzes were conducted. In addition, the ANOSIM test was used to check the differences between seasons. The Kruskal-Wallis, NMDS and ANOSIM tests were done in Past ver. 3.20 software. To elucidate the relationships between *Aeromonas* and *Legionella* spp. and their environment, Canonical correspondence analysis (CCA) together with PERMANOVA test were conducted with using R ver. 3.5.3 and RStudio ver. 1.1.463 software.

RESULTS

The physicochemical parameters of the lake water measured were averaged across all sampled depths and locations within sampling area are presented in Tab. S1. Based on physicochemical parameter values, Bray-Curtis based non-metric multidimensional scaling (NMDS) was performed (Fig. 2). The analysis allowed the samples to be grouped in terms of their physicochemical properties. First of all, there was a general trend towards grouping according to sampling season (ANOSIM R=0.4, P=0.0001). However, the exceptions constituted the samples from spring season: northern lakes: Przystań, Mamry, Dargin, Kisajno, and southern lakes: Bełdany and Śniardwy. The samples from northern lakes, sampled during spring season (Przystań, Mamry, Dargin, Kisajno), showed similarity also with northern lakes tested during summer season (Przystań and Dargin). In turn, the summer and spring samples from southern part of The Great Masurian Lakes (GML) system grouped according to geographic origin. The above indicated therefore the grouping of samples relative to the geographical location. In the case of samples taken in autumn, it was also possible to divide into samples groups from the northern part of the GML complex, as well as the southern part. A special case of a water reservoir was Lake Śniardwy - one of the southernmost of the studied area. Despite geographical location, it grouped closer to northern reservoirs - with a lower trophic status. This was

confirmed in all sampling seasons during the study period.

The trophic state index (TSI) values of the sampling sites within the GML system were defined using three variables: Secchi disc visibility, chlorophyll *a* concentration, and total phosphorus content, using Carlson's equations (Carlson 1977). Based on the nomenclature for temperate zones, the TSI values in the studied lakes ranged from oligotrophy/mesotrophy, with the lowest TSI value in the case of Mamry Lake during autumn season (TSI~39.4), to eutrophic conditions in the case of Bełdany Lake during summer season (TSI~60.5). In most cases, the trophic status of the particular lakes did not change significantly over the seasons and oscillated more or less within similar range of eutrophication status. However, there were exceptions in the cases of 4 lakes. Dargin lake mean TSI values changed from meso-eutrophic (TSI=41.9-42.8) to eutrophic (TSI=51.7) in autumn season. In Kisjano Lake TSI changed from eutrophic (TSI=52.6) to meso-eutrophic (TSI=43.8) from summer 2016 to spring 2017 (TSI=almost 50), but afterwards from spring 2017 to autumn 2017 the TSI values increased from 41.87 to 49.98 and almost reconverted. The TSI of Niegocin Lake increased from mesoeutrophic (TSI=48.2-48.5) state to eutrophic one (TSI=54.3). In turn, the TSI of Śniardwy Lake increased significantly from meso-eutrophic state (TSI=42.1) to eutrophic (TSI=50.5) in autumn. Detailed information concerning the TSI values of the studied water reservoirs in individual research seasons are presented in Tab. 1.

Real-time PCR analysis was used to quantify the presence of *Legionella* spp., *Aeromonas* spp. and *Aeromonas hydrophila* in the studied lake water samples - the results are presented in the Figs. 3 to 5. *Legionella pneumophila* was not detected in none of the studied lake. The Kruskal-Wallis test showed that the differences in the abundances of the *Legionella* spp. and *Aeromonas* spp. between individual lakes were statistically significant ($P \leq 0.0001$). Based on the number of analyzed bacteria in all studied samples, Bray-Curtis based non-metric multidimensional scaling (NMDS) was conducted (Fig. 6). The grouping of samples was observed in relation to the research season (ANOSIM R=0.41, P=0.0001) and in terms of geographical location (ANOSIM R=0.2, P=0.01).

To evaluate the relationship between the water properties of the lakes at the sampling sites and the presence of *Legionella* spp. and *Aeromonas* spp., a Canonical correspondence analysis (CCA) was applied (Fig.7). The CCA1 explains 72% of variability, while CCA2 explains 2%. Additionally, the PERMANOVA analysis was performed to check whether the model and predictors are statistically significant. The analysis showed that the amount of *Legionella* spp. is significantly positively influenced by chlorophyll *a* concentration, total phosphorus and nitrogen amount. Moreover, the dissolved

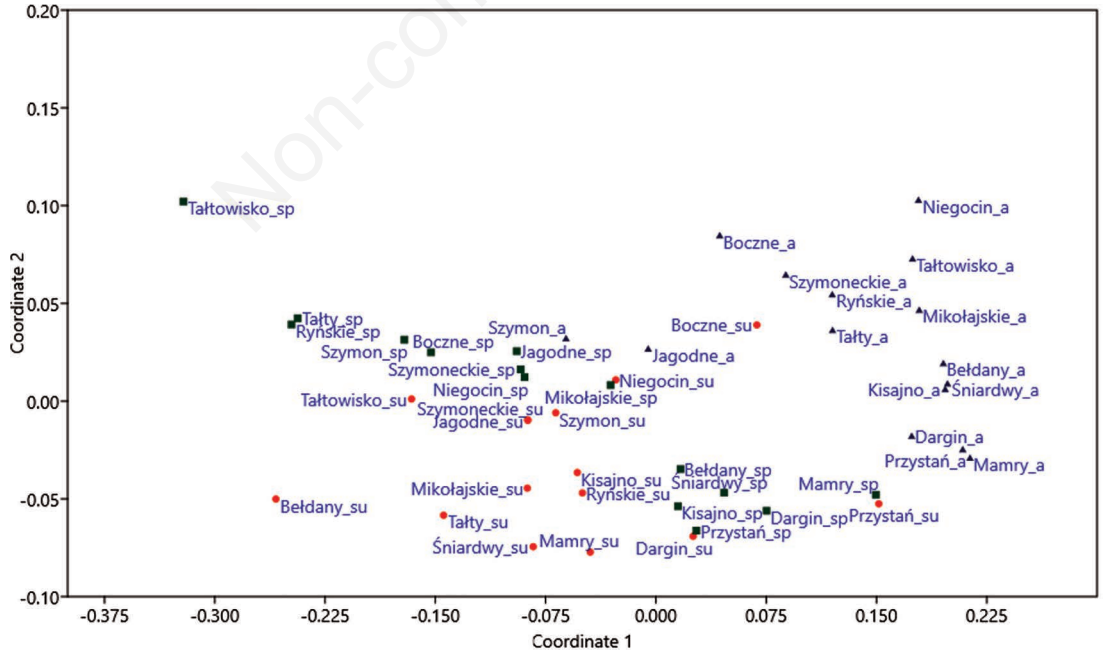

Fig. 2. The Bray-Curtis based non-metric multidimensional scaling (NMDS) of physicochemical water parameters. Stress value = 0.061. Suffixes added to sampling locations (lake's names) and marks indicate the studied seasons: _su and red dots, summer; _sp and green squares, spring; _a and dark blue triangles, autumn.

organic carbon concentration and conductivity had a positive moderate influence on *Legionella* spp. numbers in water of Great Masurian Lakes system. The amount of *Aeromonas* spp. and *Aeromonas hydrophila* was positively correlated with temperature, ammonium concentration and water transparency.

DISCUSSION

Based on the results of CCA analysis our studies have shown that there were clear visible relations between the presence of *Aeromonas* and *Legionella* spp. and water eutrophication variables, such as phosphorus, nitrogen,

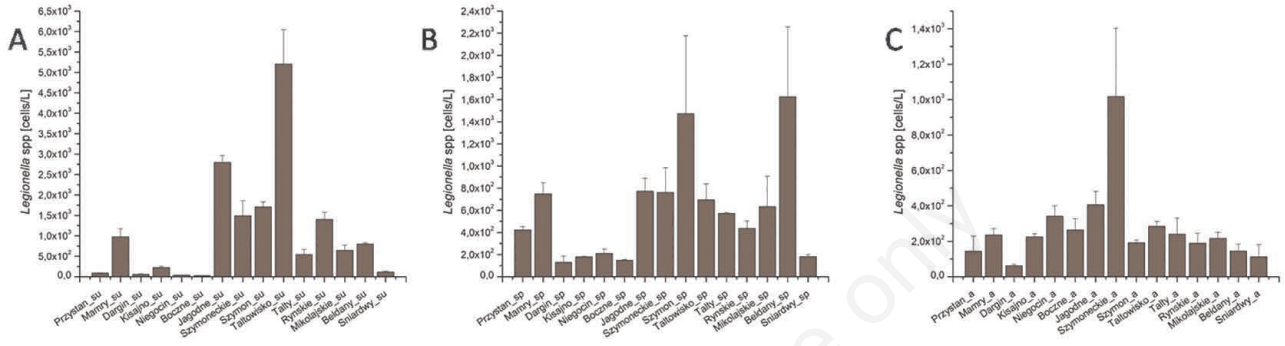

Fig. 3. *Legionella* spp. cells concentration in studied lakes during summer (a), spring (b) and autumn (c) seasons. Suffixes added to sampling locations (lake's names) indicate the studied seasons: _su, summer; _sp, spring; _a, autumn.

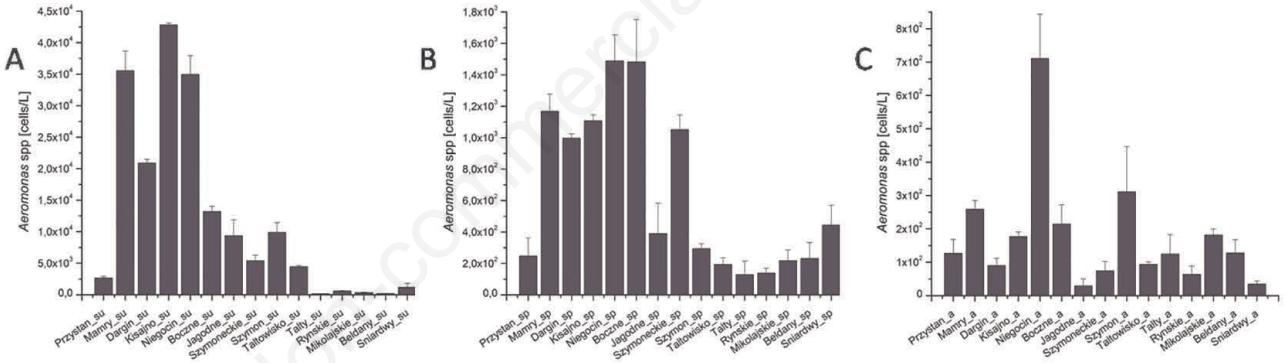

Fig. 4. *Aeromonas* spp. cells concentration in studied lakes during summer (a), spring (b) and autumn (c) seasons. Suffixes added to sampling locations (lake's names) indicate the studied seasons: _su, summer; _sp, spring; _a, autumn.

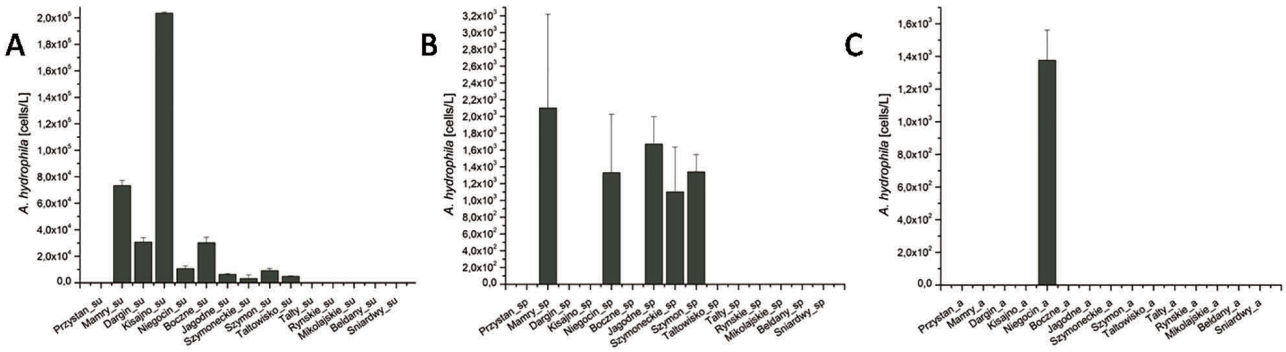

Fig. 5. *Aeromonas hydrophila* cells concentration in studied lakes during summer (a), spring (b) and autumn (c) seasons. Suffixes added to sampling locations (lake's names) indicate the studied seasons: _su, summer; _sp, spring; _a, autumn.

ammonia and chlorophyll concentrations, conductivity, turbidity, and water transparency, that was presented by the results of CCA analysis. This demonstrates that further eutrophication of the studied lakes of the GML system can cause serious microbiological risks, both to animals and human health, which cannot be neglected.

About one hundred years ago, the Great Masurian Lakes were classified as oligotrophic water reservoirs (Gieysztor and Odechowska, 1958). During the 1950s eutrophication began to accelerate significantly as a consequence of human activity and speeding up of the urban development (Kauppinen, 2013). Intensification of agriculture, tourism development and expansion of towns and villages began significant changes in the surroundings of the Great Masurian Lakes system as well in the water reservoirs themselves (Siuda *et al.,* 2020). More than half of the lakes were supplied with municipal and camping sewage (Ozimek and Kowalczewski, 1984). According to Kajak *et al*. (1975) during the 1970s the Mikołajskie Lake was highly eutrophicated. During the 1980s the southern lakes of the system became hyper-eutrophicated. Regarding northern lakes, with short-term exceptions, their trophic state was at the meso-eutrophy level. The main sources of nutrients in their case are tourism and agriculture (Wołos *et al*., 2009). In the 1990s especially in the southern part of Great Masurian Lakes system trophic state began to descend, because of modernization of sewage treatment plant that was discarding the effluent and limiting the inflow of industrial and agricultural wastewater to Niegocin Lake. Since about 2005 the oligotrophication decelerated and subsequently even reversed. Therefore, we are still dealing with the effects of accelerated eutrophication (Kauppinen, 2005).

The GML system consists of a complex system of water reservoirs with a wide variety of physicochemical characteristics. The grouping of similar sampling points in terms of their physicochemical properties simultaneously with their proximities in geographical terms was clearly visible. Sampling site located within Śniardwy Lake was an exception. It was similar to the group of northern lakes in terms of physicochemical properties and in terms of *Legionella* and *Aeromonas* spp. content profile, even though it was one of the most southern sites studied within the research area. This result may be due to the significant remoteness of the studied sites from the rest and the location within a water reservoir with different characteristics than the neighboring lakes.

The similarities between water reservoirs in term of physicochemical profile (Fig. 2), at the same time in terms of the amount of potentially pathogenic microorganisms (Fig. 6) indicate the significance of environmental factors related to eutrophication in occurrence of studied bacteria (Anza *et al*., 2014). In our studies we observed that in case of Śniardwy and northern lakes. Śniardwy Lake is shallow, large lake that, despite the location and strong anthropopressure is in meso-eutrophic state and its

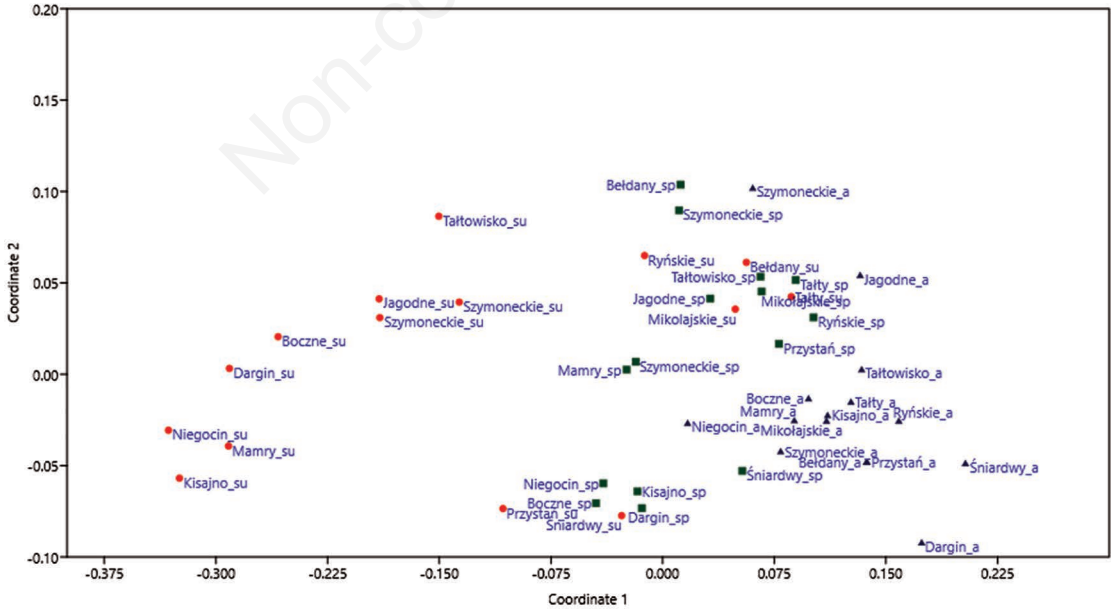

Fig. 6. The Bray-Curtis based non-metric multidimensional scaling (NMDS) of *Legionella* spp., *Aeromonas* spp. and *A. hydrophila* number. Stress value = 0.057. Suffixes added to sampling locations (lake's names) and marks indicate the studied seasons: _su and red dots, summer; _sp and green squares, spring; _a and dark blue triangles, autumn.

physicochemical characteristics are similar to those characterizing northern, less eutrophicated lakes (Siuda *et al.,* 2020), what was demonstrated in our studies by non-metric multidimensional scaling (NMDS) analysis. Additionally, NMDS analysis showed that the lakes located next to each other have a similar profile of potentially pathogenic bacteria: *Legionella* and *Aeromonas* spp. This suggests that water is a mediator in the transmission of pathogens.

The ANOSIM test showed that there are significant differences regarding the presence of pathogens in the different sampling seasons. In general, the largest amount of *Legionella* and *Aeromonas* spp. was observed in summer period, at the peak of the growing season that was also the peak of the tourist season. That corresponds with the previous study (Siuda *et al*., 2020), where during the peak of tourist season, the substantial amount of pollutants is generated by the yacht ports and runoffs more than usual.

The results of CCA analysis showed that the presence of potentially pathogenic *Legionella* and *Aeromonas* was associated with factors being indicators of the trophic states of the studied lakes (chlorophyll *a* concentration, total phosphorus amount, total nitrogen amount, ammonium concentration, water transparency). Eutrophication of aquatic environments, which is manifested by increases in water productivity resulting from nutrient enrichment, is one of the most visible examples of negative human impacts to the biosphere (Smith *et al*., 1999). Nutrient enrichment interacts with the ecological stability of a system and determines the presence of other contaminants, including infectious disease agents (Smith and Schindler, 2009). The characterization of the trophic state of an aquatic ecosystem includes the definition of biogenic substances, mainly phosphorus, chlorophyll *a* concentration and water transparency (Petrucio *et al*., 2005). The use of three variables to determine the trophic status in our studies was justified because it is not always possible to determine the trophic state of a studied lake with a single variable. For example, in the case of Mamry Lake, we observed very low chlorophyll *a* concentrations indicating an oligotrophic conditions of a lake, while the turbidity was quite high and did not result from a large amount of phytoplankton; moreover, both the transparency and phosphorus content pointed to a mesotrophic state.

Chlorophyll *a* concentration is an indicator of the abundance of photosynthetic organisms. In lake ecosystems, photosynthetic organisms are the base of the food chain and influence the trophic state. Nutrient content is one of the major factors that regulate photosynthesis. Hence, these parameters were considered when determining the index values of the trophic state. Previous study reported that the trophic conditions of lake water had significantly positive effects on the total

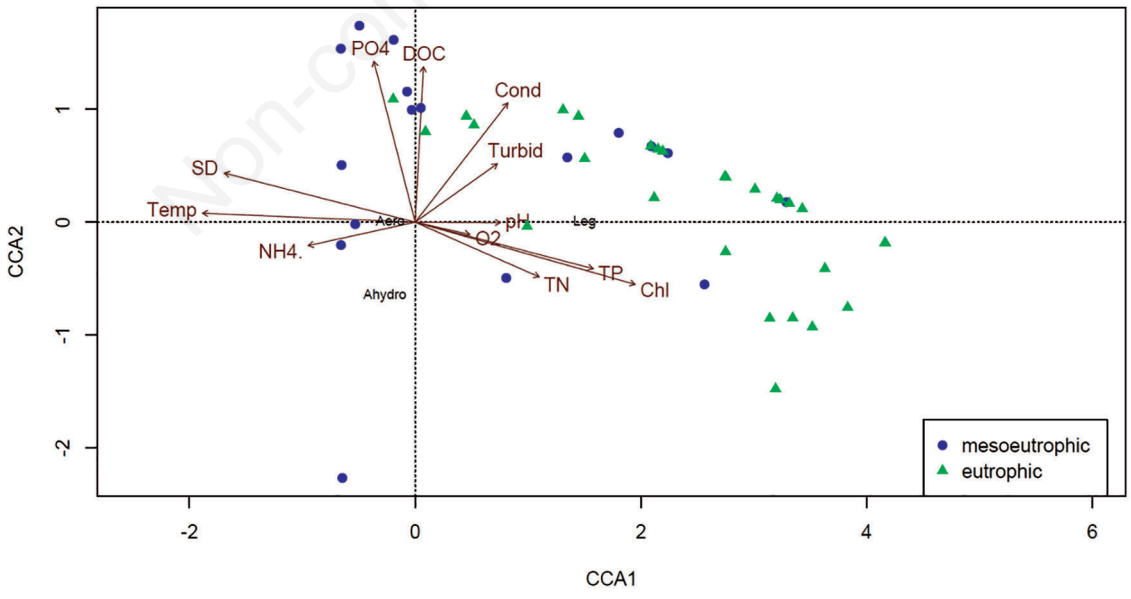

Fig. 7. Canonical correspondence analysis (CCA) results. The CCA1 explains 72% of variability, while CCA2 explains 2%. Cond, conductivity (µs cm^{-2}); DOC, dissolved organic carbon (mg L); PO_4, orthophosphates amount (µg L^{-1}); Chl, chlorophyll *a* concentration (µg L^{-1}), TP, total phosphorus amount (µg L^{-1}); TN , total nitrogen amount (µg L^{-1}); Turbid, turbidity (NTU); Temp, temperature (°C); O_2, oxygen concentration (mg O_2L^{-1}); SD, Secchi disc visibility [m]; NH_4^+ , ammonium concentration (mg L^{-1}); Leg, *Legionella* spp. number (cells L^{-1}); Aero, *Aeromonas* spp. number (cells L^{-1}); A. hydro, *Aeromonas hydrophila* (cells L^{-1}).

numbers of bacteria, HNF (Heterotrophic Nanoflagellates) and other biotic variables of the Great Masurian Lake system (Chróst and Siuda, 2006).

What deserves attention is the effect of ammonium on the amount of *Aeromonas* spp. and *Aeromonas hydrophila*. Ammonium is a widespread pollutant in aquatic ecosystems originating directly and indirectly from human activities, which can strongly affect the structure and functioning of the aquatic foodweb and microbial structure (Leoni *et al*., 2018).

In aquatic environments, autochthonous microflora can use trace amounts of nutrients. In the case of allochthonous bacteria, growth deceleration is usually observed after entering nutrient-poor aquatic environments. In this adaptive phase, bacteria adjust to new environmental conditions. The more oligotrophic the environment, the longer the adaptation time because of the time needed to reorganize the enzyme systems in the microbial cells (Jones *et al.,* 2004). Therefore, the higher the eutrophic status, the shorter the adaptation and growth restraint time (Nazari-Sharabian *et al.,* 2018). Consequently, environments more abundant in nutrients promote the survival and growth of microflora. For this reason, the pollution of water bodies, introduced with surface runoffs, related to human recreational activity, contributes not only to the introduction of allochthonous pathogenic bacteria but also to their propagation.

Under unfavorable environmental conditions, bacterial cells are able to survive by inhibiting their growth rates and slowing down their metabolism. Different survival strategies of allochthonous bacteria in aquatic environments allow for the extension of survival time, e.g., after temperature decreases. Bacterial cells pass into the VBNC *(viable but not culturable)* state that is characteristic for living but non-cultivable cells, and these cells also constitute a threat to public health. Bacteria being in the VBNC state in fact are still virulent (Colwell 2000). *Legionella* spp. are an example of bacteria, which commonly enter a VBNC state in water environments. This state is induced often by nutrient starvation. There is a huge threat for water reservoirs safety, because increased trophic state caused by the nutrient supply can cause a sudden multiplication of these bacteria through resuscitation (Edagawa *et al*., 2015; Garner *et al*., 2018). VBNC state formation is one of reason for using a sensitive molecular biology technique, which is real-time PCR. This method is also justified due to the fact that only a small part of the bacteria community (approximately 0.1%) inhabiting the environment is cultivable (Cho and Giovannoni, 2004; Dupont *et al*., 2014). Moreover, several studies show that molecular methods are an advantage over classic cultivation methods (Lleo *et al*., 2005; Wade, 2011; Rhoads *et al.,* 2012).

The relation between temperature and the presence of potentially pathogenic *Aeromonas* bacteria also deserves attention. Similar relationships can be observed in other studies (Jin *et al*., 2018). This can be particularly dangerous in the context of climate change and increasing surface water temperatures (Mujere and Moyce, 2016).

Because nutrient loading, and hence eutrophication, will become more severe and widespread, eutrophication will continue to be an important factor in the etiology of human diseases. Eutrophication is, in fact, a problem that is very difficult to reverse. In freshwater, nutrients that are stored in sediments can be rapidly recycled (Johnson and Carpenter, 2008). This means that de-eutrophication is a very slow process and a strongly eutrophicated water reservoir can be a microbiological threat for a long time. The problem of dependence between trophic status and the presence of pathogenic bacteria is far-reaching and requires attention. This is particularly motivated by the fact, that previous studies have shown that *Legionella* and *Aeromonas* spp. infection is associated with the ability to thrive and persist in environment as a result of environmental selection. That is why we have referred the presence of *Legionella* and *Aeromonas* spp. to the environmental conditions.

The correlation between the studied bacteria and environmental factors related to anthropopressure causing eutrophication indicated, that there is justified urgent need of monitoring microbiological quality of natural waters and considering different species of microorganisms in lakes which may cause sanitary and health problems.

CONCLUSIONS

The study showed, that factors related to the eutrophication of surface waters are related to the presence of potentially pathogenic bacteria: *Legionella* and *Aeromonas* spp. That is particularly important in context of the ongoing eutrophication of surface water. This is the first study aimed to monitor the presence of those microorganisms over several study seasons in the waters of the Great Masurian Lakes system. Our findings suggest that further degradation of the aquatic ecosystem due to eutrophication constitutes a serious threat to human and animal health.

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