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THE RELATIONSHIP BETWEEN THE PRESENCE OF *HELICOBACTER PYLORI* AND THE COMPOSITION OF IONIC AND ORGANIC MICROELEMENTS IN DRINKING WATER FROM CRACOW

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Although the natural niche for *H. pylori* (*Hp*) is the human stomach, for widespread infection to occur this microorganism may need to survive in the external environment. Molecular techniques such as polymerase (PCR) have revealed the presence of *Hp* DNA in water, indicating that this environment could act as a reservoir for this bacterium. The aim of this study was to analyse the occurrence of *Hp* in tap water from Cracow and to examine the relationship between 26 parameters and the presence of *Hp* DNA due to the lack of such information related to this issue in Poland. Additional aim of this study was to determine whether the correlation between *Hp* DNA detection and seasonal changes of water quality in 379 water samples collected from various water treatment plants (WTPs), could be found. Water samples were subjected to PCR for *glmM* and *cagA* genes. Ionic and organic composition of microelements were determined in accordance to Polish and ISO standards. The data obtained from tests show that 212 (55.96%) objects were *Hp* DNA (*glmM*) positive and among them 145 (68.40%) waters samples revealed expression of *cagA*. Linear Discriminant Analysis and Principal Component Analysis were used and provided that the selected variables ($p < 0.05$): colour, pH, conductivity at 25ºC, chlorides, nitrites, nitrates, phosphates, chlorates, chlorites, sulphates, free chlorine, sodium, magnesium, potassium, calcium, organic carbon, trichloromethane, bromodichloromethane, dibromochloroethane, total iron, ammonium ion, and ∑TMHs distinguished the water samples that contain *Hp* DNA and do not contain *Hp* DNA. We conclude that the ionic and organic composition of microelements in water might influence the presence of *Hp*. Thus, determination of the selected microelements may indirectly indicate or sometimes predict the presence of *Hp* in drinking water.

Key words: *Helicobacter pylori, tap water, microelements, cytotoxin-associated gene A, glmM gene, seasons, Principal Component Analysis, Linear Discriminant Analysis*

INTRODUCTION

Helicobacter pylori (*Hp*) is a pathogen of constant interest to researchers who are constantly looking for answers to many questions. One is the question: 'How does infection occur and what is the source of it?'. Although the natural niche for *Hp* is the human stomach, for widespread infection to occur the organism may need to survive in the external environment (1). Numerous studies were conducted to explain pathogenesis of human infection by this bacterial microorganism. However, the epidemiological aspect concerning the transmission of *Hp* infection and its multifactorial determinants still should be elucidated.

Previous studies, have indicated that water could be an important source of *Hp* contamination (2-16). On July 2021, United States Environmental Protection Agency (USEPA) published the Draft Fifth Contaminant Candidate List (CCL 5). The CCL is a list of contaminants that are currently not subject to any proposed or regulated national primary drinking water regulations but are known or anticipated to occur in public water systems. Contaminants listed on the CCL may require future regulation under the Safe Drinking Water Act (SDWA). The Draft CCL 5 includes 66 chemicals and 12 microbes. *Hp* along with the microbial contaminants are significant pathogens for public health due to their association with multiple diseases (17).

The infection with *Hp* (classified as a group I carcinogen in humans by the International Agency for Research on Cancer) is considered as one of the most significant risk factor for gastric cancer. Additionally, a list published in 2017 by the WHO includes *Hp* among microbes for which new antibiotics are urgently needed (18). Our present work was aimed to determine whether water plays a role in the spread of the *Hp* infection and whether elements found in tested samples of water may influence the development of *Hp*. To date, there has not been any research conducted in Poland to identify the presence of *Hp* in water.

By determining the concentrations of examined ions and the possible correlation between the presence or absence of *Hp*

contamination of water we wanted to provide an insight into possible survival of this microorganism in environmental conditions. Furthermore, we attempted to define the role of microelements in the mechanism of the inhibition or stimulation of *Hp* proliferation. The fastidious nature of the *Hp* and the difficulties in isolating it from milieu do not provide sufficient evidence on how this bug can survive in water environment and how water can severe a source of transmission of this microorganism (19).

Hp is a Gram-negative bacillus, unipolar flagellated, spiral-shaped, both enzymes urease and catalase positive. Under conditions of stress (20) this spiral form can adopt a coccoid morphology entering a viable but non-cultivable (VBNC) state, in which the bacterium is unable to grown on agar plates by using conventional culture methods (21). The VBNC cells retain membrane integrity and contain undamaged genetic information. It was proposedly that by entering this state, bacteria can survive in this environment (22).

A number of studies concerning drinking water have identified *Hp* in water pre- and post-chlorination (23). *Hp* could survive disinfection practices used in drinking water treatment in the VBNC and would not be detectable by culture methods. Moreno *et al.* (24) have shown that *Hp* could survive disinfection practices that are commonly used in drinking water treatment where *Hp* are found in the VBNC state. However, they found that culture of *Hp* was lost after 5 min in water despite free chlorine levels of 0.96 mg/L (24). Baker *et al.* have reported that *Hp* was more resistant to low levels of free chlorine than *Escherichia coli* or *Campylobacter jejuni* (25). Orta de Velasquez *et al.* (26) conducted water disinfection tests using ozone and chlorine to evaluate their effects on VBNC *Hp* cells. They found that bacterial inactivation procedure using ozone (O_3) and chlorine resulted in the VBNC *Hp* membrane damage. This study provide an evidence that ozone can destroy VBNC *Hp* in aqueous solution more effectively than chlorine (26).

It has been documented that the bacterial culture, the immunological methods and molecular methods would allow to detect *Hp* in the aquatic environment (19). The PCR methods have focused on targeting the urease C gene (*ureC*) which has been renamed *glmM* by de Reuse *et al.* in 1997 (27). The *glmM* is a highly conserved housekeeping gene which has been used for *Hp* identification not only in gastric biopsies but also in water samples (11, 12, 14, 16, 28-30). The *glmM* gene encodes phosphoglucosamine mutase, an enzyme catalyzing the interconversion of glucosamine-6-phosphate into glucosamine-1-phosphate, which is subsequently transformed into N-acetylglucosamine (31). The advantage of the glmM gene over other genes detectable in gastric biopsies of *Hp*-infected individuals can be due to a higher degree of sensitivity and specificity which can detect 10 – 100 *Hp* cells in the clinical specimens, being superior over histopathological examination (32).

One of the most well characterized factors of *Hp*'s virulence is cytotoxin associated gene A (*cagA*) which encodes an immuno-dominant bacterial protein *CagA* with a variable molecular weight (120 kDa to 140 kDa). The *cagA* gene is located on the cag pathogenicity island (*cagPAI*) (33). The *cagPAI* is not present in all *Hp* strains, although it contains 27 to 30 genes, encoding a type IV secretion system (T4SS) (34-36). CagA is injected into the cytoplasm of the gastric epithelial cells *via* the type IV secretion system, where it can impair the signal transduction system, modify cellular functions and induce abnormal proliferation of epithelial gastric cells and can alter cell phenotypes at a relatively early stage of the gastric carcinogenesis process (37).

Acquisition of *Hp* usually happens during childhood through intra-familial transmission. The other most recognized ways of this bug transmission is the person-to-person spread, but oral-oral, and fecal-oral transmission have also been described (38-40). Interestingly, Dey *et al.* (8) have provided an evidence to support the hypothesis that amoebae and perhaps, other free-living protozoa contribute to the replication and persistence of *Hp* by providing a protected intracellular microenvironment for this pathogen to persist in natural aquatic environments and engineered water systems, thereby *Hp* may potentially use amoeba as a carrier and a vector of transmission (8).

Another issue being recently explored is the search for the relationship between season and *Hp* occurrence in water and environmental samples (4, 5, 15, 41, 42). Studying the correlation between season and the occurrence of *Hp* in water samples may be important in conjunction with the frequency of *Hp* infection in dyspeptic patients.

The aim of our present study was to analyze the occurrence of *Hp* in tap water from the city of Cracow. Therefore, we examined the relationship between water quality and presence of *Hp* DNA due to the absence of information related to this issue in Poland. Additional aim of this study was to determine whether a correlation exists between year seasons and *Hp* DNA detection in tap water samples collected from water treatment plants (WTPs).

MATERIALS AND METHODS

Sample collection

Production of drinking water for the city of Cracow was conducted in four WTPs: WTP Raba, WTP Rudawa, WTP Dlubnia, WTP Bielany (Sanka river). Quality of water produced for the Cracow agglomeration meets the quality standards obligatory in Poland and the European Union (EU) recommendations. The sources of raw water for individual WTP are rivers: Sanka for WTP Bielany, Dlubnia for WTP Dlubnia, Rudawa for WTP Rudawa, Raba for WTP Raba (*Fig. 1*).

The search for *Hp* DNA in 379 tap water samples was carried out between July 2012 and January 2016 in Cracow. The water samples were collected every month, each year.

We collected water samples from 52 sampling points including: designated schools, universities, nurseries, kindergartens, shops, gas stations, congress centers, state offices, hospitals, medical clinics, service points, which were located within the area supplied by the WTPs (*Table 1*).

Detection of Hp DNA by amplified glmM and cagA in water samples

For the water collection, 1 L of water was taken aseptically to the sterile glass bottles, all samples then being transported at 4ºC. First, for each sample 1L water was concentrated by centrifugation 121 × g for 5 min and later on the centrifugation was repeated once again at 8000 × g for 20 min. Sediments were resuspended in PBS buffer and concentrated by centrifugation 10000 × g for 5 min then aliquots were stored at –20ºC (43, 44). These aliquots were used later to extract DNA by Genomic mini AX bacteria (A&A Biotechnology, Gdynia, Poland) in accordance with the

Table 1. Number of water collection points in Cracow at sites located within the area supplied by the given WTPs.

WTP	Number of sampling points
WTP Bielany (Sanka river)	5
WTP Rudawa	9
WTP Dlubnia	5
WTP Raba	33

manufacturer's recommendations. The quality and the amount of genomic DNA were measured by using Nanodrop ND-1000 (ThermoFisher, Waltham, MA, USA). All PCR reactions were carried out employing Promega PCR reagents (Promega Corporation, Madison, WI, USA) and 100 ng DNA isolated from water samples. The PCR mixture was amplified in thermal cycler Biometra T3 (Biometra, Berlin, Germany). Specific primers for detection *cagA* and *glmM* (45-49) were synthesized by Sigma-Aldrich (Sigma Aldrich, St. Louis, MO, USA) and the primers sequences are shown in *Table 2*.

PCR products were separated by electrophoresis in 2% agarose gel containing 0.5 µg/mL ethidium bromide and then visualized under UV light. Location of predicted PCR product was confirmed by using O'Gene Ruler 50 bp DNA ladder (ThermoFisher, Waltham, MA, USA) as standard marker. We used *Hp* ATCC 43504 as a positive control and the sterile and deionized water as a negative control.

Amplicons from the analyzed water samples were sequenced in a ABI-PRISM 3500 (ThermoFisher, Waltham, MA, USA), using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher, Waltham, MA, USA). The Basic Local Alignment Search Tool (50) was used for processing the sequencing data and to identify the *cagA* gene.

Physicochemical and microbiological analysis

All parameters were examined according to the regulation of the Polish Ministry of Health (dated November 13th, 2015) on the quality of water intended for human consumption due to the relevant polish standards (51-52). A summary of the 26 parameters in the tested water samples, the units and the corresponding standards are presented in *Table 3*.

Statistical analysis of the data

All statistical analyses were conducted in Matlab R2021a software from Mathworks company. Differences in data values were considered significant at $p < 0.05$.

To determine whether there is a correlation between the 26 elements and the presence of *Hp*, the Principal Component Analysis (PCA) and the Linear Discriminant Analysis (LDA) were used. These unsupervised learning methods belong to one of two basic groups of chemometric algorithms enabling the detection of the structure of a set of objects, recognition of similar cases that constitute subsets of the analyzed space and outliers. PCA is an algorithm with great research potential. It allows to reduce the size of the data space by transforming the correlated

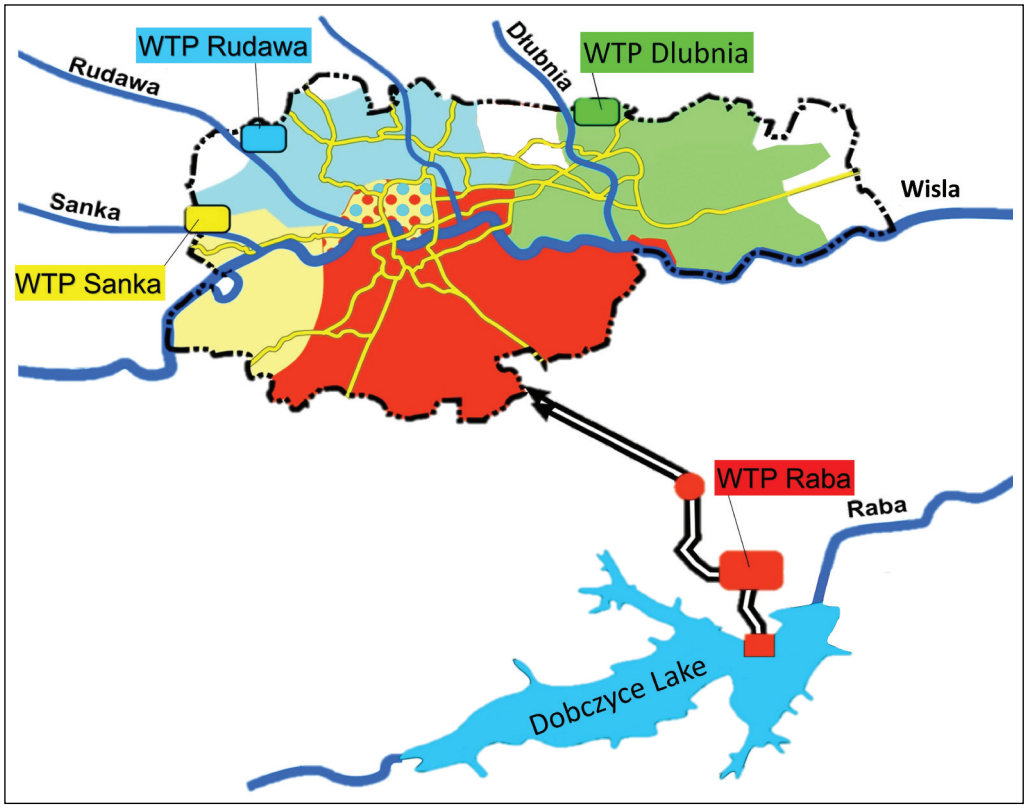

Fig. 1. Scheme of sources supplying water for the areas of the city of Cracow from particular water intakes. Modified based on materials obtained from Waterworks Cracow.

Table 2. Oligonucleotide primers for detection *cagA* and *glmM* by PCR, annealing temperature and size of PCR products employed in the experimental protocol.

Gene	Primer Sequence	Size of PCR product (bp)	Annealing temperature (ºC)
cagA	forward: ATA ATG CTA AAT TAG ACA ACT TG reverse: TTA GAA TAA TCA ACA AAC ATC AC	298	60
glmM	forward: AAG CTT TTA GGG GTG TTA GGG GT reverse: AAG CTT ACT TTC TAA CAC TAA CG	294	60

Table 3. The list of 26 parameters in the tested water samples, unit and the relevant norm.

	Parameter	Unit	Standard
1	total number of microorganisms at 22ºC after 72 h	CFU/1mL	PN-EN ISO 6222:2004
2	color	mg/l Pt	PN-EN ISO 7887:2012
3	pH		PN-EN ISO 10523:2012
4	conductivity at 25°C	µS/cm	PN-EN 27888:1999
5	chlorides	mg/L	PN-EN ISO 10304-1:2009
6	nitrites	mg/L	PN-EN ISO 10304-1:2009
7	nitrates	mg/L	PN-EN ISO 10304-1:2009
8	phosphates	mg/L	PN-EN ISO 10304-1:2009
9	chlorates	mg/L	PN-EN ISO 10304-4:2002
10	sulphates	mg/L	PN-EN ISO 10304-1:2009
11	free chlorine	mg/L	PN-EN ISO 7393-2:2011
12	total iron	mg/L	PB-NJL-W-02
13	aluminum	mg/L	PB-NL-W-26
14	sodium	mg/L	PN-EN ISO 14911:2002
15	ammonium ion	mg/L	PN-EN ISO 14911:2002
16	potassium	mg/L	PN-EN ISO 14911:2002
17	magnesium	mg/L	PN-EN ISO 14911:2002
18	calcium	mg/L	PN-EN ISO 14911:2002
19	fluorides	mg/L	PN-EN ISO 10304-1:2009
20	chlorites	mg/L	PN-EN ISO 10304-4:2002
21	sum of chlorates and chlorites	mg/L	PN-EN ISO 10304-4:2002
22	organic carbon OWO	mg/L	PN-EN 1484:1999
23	trichloromethane	µg/L	PB-NJL-W-06
24	bromodichloromethane	µg/L	PB-NJL-W-06
25	dibromochloroethane	µg/L	PB-NJL-W-06
26	∑TMHs (sum trihalogenomethanes)	µg/L	

experimental variables into new, orthogonal ones, i.e. principal components. The possibility of presenting the graphical structure of a multidimensional data set on a plane is often used, with little loss of information. Algorithm of the dimensionality reduction of a data space relies on projecting the data to the space with a smaller number of dimensions, to best preserve the data structure. The main rule is to maximize the variance of each new variable. Taking into account the optimal number of principal components it allows effective data analysis. PCA is used to reduce the information contained in many variables to a small number of explanatory dimensions in order to explore and identify the structure of the dataset. It is possible to discover new dependencies or confirm observations already known.

LDA belongs to a group of algorithms that enable exploration of a data set using a qualitative dependent variable. It allows to examine differences between two or more groups by analyzing several variables, simultaneously. The task of the method is to decide which explanatory variables best divide a given set of cases into naturally occurring groups, described by the response variable. LDA defines the rules for assigning multidimensional objects to subsets with the minimum possible classification errors. The method considers multiple variables to see which of them contribute best to the group discrimination. The variables used to distinguish between the groups are called discriminant variables. In order to detect whether an object belongs to a class, linear functions are used. The examined case is assigned to the class for which the discriminant function achieves the highest value.

In this work, both PCA and LDA were used as statistical methods to describe and interpret intergroup differences.

RESULTS

Fig. 2 shows representative PCR products for gene *glmM* and gene *cagA* in the selected water samples.

The data obtained from water distribution system of Cracow area from the PCR method (tests) showed that 212 (55.96%) objects were *Hp* DNA (*glmM*) positive and among them 145 (68.40%) waters samples revealed expression *cagA* (*Table 4*). Sequencing of all amplicons showed that the sequence of all of them was 98 – 100% similar to a fragment of *Hp cagA* gene sequence.

Table 4 describes number of water samples containing *Hp* (1) DNA and not containing *Hp* (0) DNA, based on expressing genes *glmM* and *cagA* in drinking water sampling points at WTPs. Based on the expression of gene glmM (*Hp* DNA), the PCA and LDA analyses were performed.

Analysis of the Hp DNA presence in drinking water intake points at WTPs

At the beginning, the PCA was performed for the entire dataset considering all 26 variables presented in *Table 3*.

The PCA method did not differentiate samples with *Hp* (1) DNA from samples without *Hp* (0) DNA (*Fig. 3*). Instead, the data presentation in *Fig. 3* attempts to describe the variability of the PC, and the projection of points on PC1-PC3. Since all variables do not allow for inference relevant to the considered problem, thus, we have decided to check whether it was possible to distinguish the individual WTP based on the parameters measured in the water samples.

The WTPs can be considered separately because they form clusters that can be seen in *Fig. 4*. Only points obtained from WTP Raba and WTP Rudawa & WTP Raba cannot be distinguished and therefore, they were considered as one group. We then have checked which variables differentiate samples

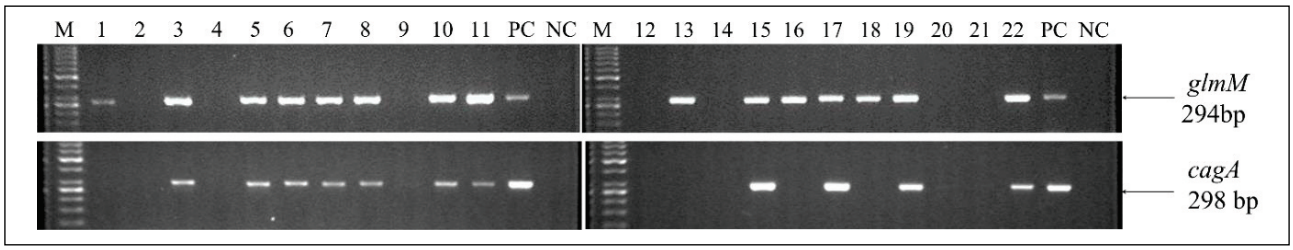

Fig. 2. Representative results for PCR products (*glmM* and *cagA*) generated from 22 selected water samples. Lanes: M - DNA size marker; 1 – 22 - representative water samples; PC - positive control (*Hp* ATCC 43404), NC - negative control (water without DNA), lines 3, 5 – 8, 10, 11, 15, 17, 19, 22 show positive result for *glmM* and *cagA*, line 1, 13, 16, 18 show positive result for *glmM* and negative result for *cagA*, line 2, 4, 9, 12, 14, 20, 21 show negative result for *glmM* and *cagA*.

Table 4. Number of water samples and frequency of *Hp* DNA occurrence (*glmM* and *cagA*) in WTPs.

	WTP	Number of water samples	*Hp* (1) DNA		*Hp* (1) DNA%		*Hp* (0) DNA	*Hp* (0) DNA %
			glmM	*cagA*	*glmM*	*cagA*		
1	**Bielany (Sanka river)**	36	16	12	44.44	75.00	20	55.56
2	**Rudawa**	89	56	39	62.92	69.64	33	37.08
3	**Dlubnia**	40	28	16	70.00	57.14	12	30.00
4	**Raba**	186	97	70	52.15	72.16	89	47.85
5	**Rudawa & Raba**	28	15	8	53.57	53.33	13	46.43
	Sum	379	212	145			167	

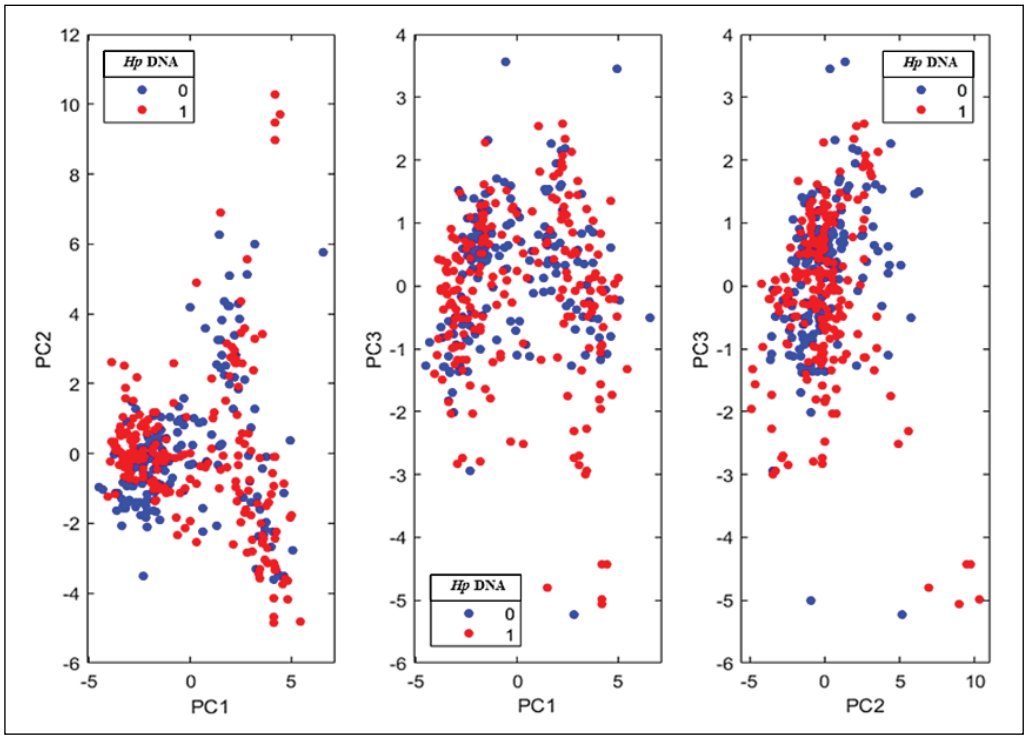

Fig. 3. Projection of the points representing all water samples and all experimental variables on PC1/PC2, PC1/PC3 and PC2/PC3 planes.

containing and not containing *Hp* DNA for each of the WTP separately with WTP Raba alone, and WTP Rudawa and WTP Raba considered together. The p-values are given in *Table 5*. Having these differentiating variable examined variables, the PCA has been employed for each WTP.

Fig. 5 was created using 3 variables: magnesium, trichloromethane and bromodichloromethane for which $p < 0.05$. These variables allow water samples to be separated to those containing *Hp* (1) DNA and not containing *Hp* (0) DNA. LDA was performed based on the same selected variables in the next step. LDA correctly differentiated 29 (80.6%) of all samples from WTP Bielany (Sanka river), including 15 (71.4%) of the correct samples labeled *Hp* (0) DNA and 14 (93.3%) of the correct samples labeled *Hp* (1) DNA (*Table 6*). It was concluded that

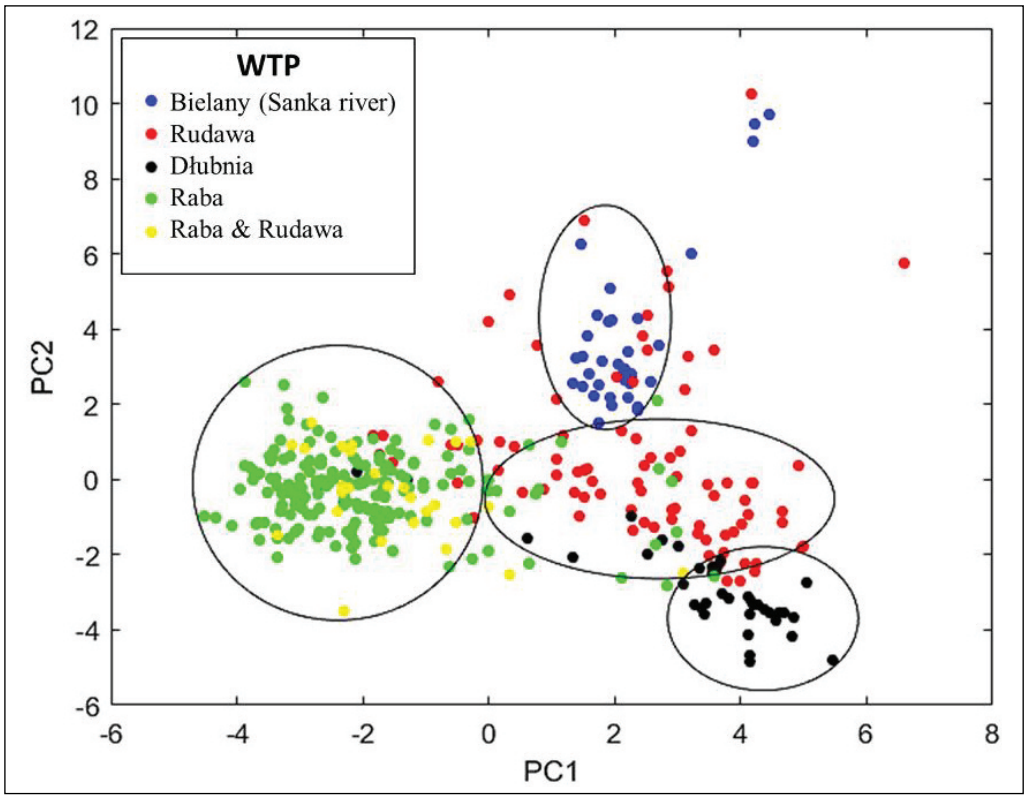

Fig. 4. Projection on the PC1/PC2 plane of points representing all water samples and all variables in individual WTP. The subsets are marked with ellipses.

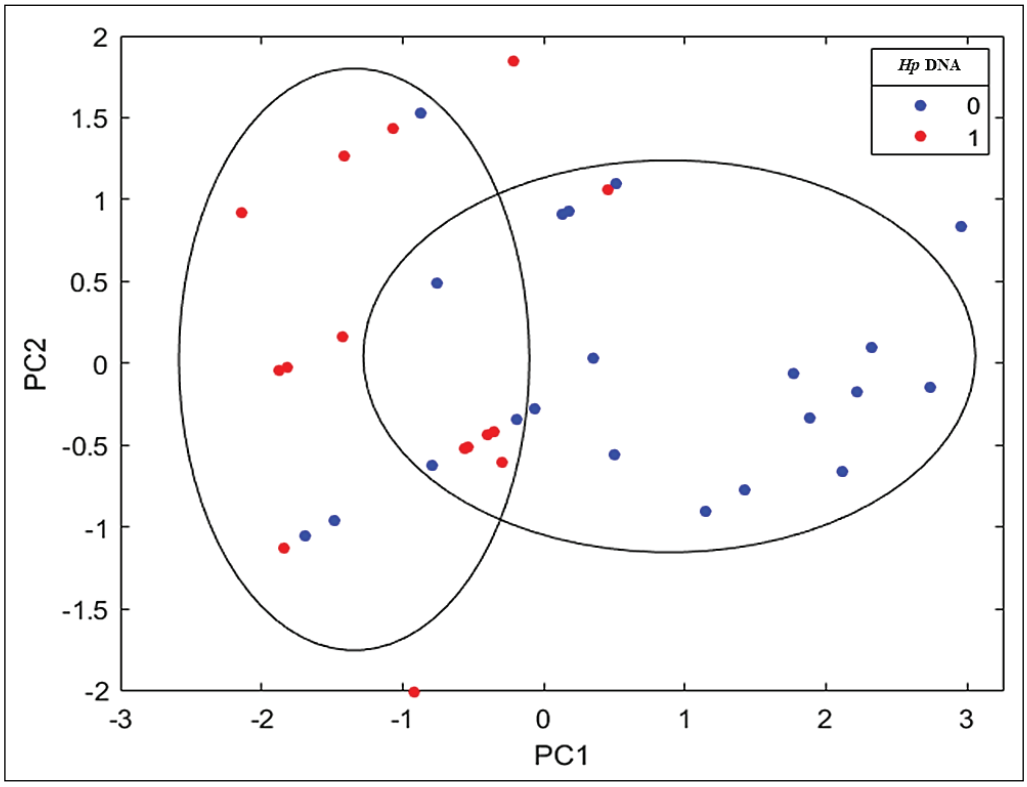

Fig. 5. Projection of points representing the water samples from WTP Bielany (Sanka river) on the PC1/PC2 plane. The sets of water samples marked with two ellipses.

based on the selected variables, LDA correctly recognizes *Hp* (1) DNA and *Hp* (0) DNA with the accuracy above 80%.

Fig. 6 was created using 3 variables: color, nitrites and total organic carbon for which $p < 0.05$. These variables separate water samples that contain *Hp* DNA and not contain *Hp* DNA. LDA was performed based on the same selected variables in the next step. LDA correctly differentiated 64 (71.9%) of all WTP Rudawa, including 10 (31.2%) of the correct samples labeled *Hp* (0) DNA and (54) 94.7% of the correct samples labeled *Hp* (1) DNA (*Table 7*). It was concluded that using the selected variables, LDA correctly recognizes samples containing *Hp* (1) DNA. Unfortunately, most of the negative samples were classified as positive.

Fig. 7 was created using 3 variables: chlorites, phosphates and sulfates for which $p < 0.05$. These variables separate water samples containing *Hp* (1) DNA from those that do not contain *Hp* (0) DNA. For WTP Dlubnia, when selecting the sample differentiating variables, alpha = 0.1 was assumed. LDA was performed based on the same selected variables in the next step. LDA analysis correctly differentiated 30 (75.0%) of all WTP Dlubnia, including 5 (41.7%) of the correct samples labeled *Hp* (0) DNA and 25 (89.3%) of the correct samples labeled *Hp* (1) DNA (*Table 8*). It was concluded, that based on the selected variables, LDA correctly recognizes *Hp* (1) DNA. Unfortunately, most negative samples are classified as positive.

Fig. 8 was created using 10 variables: color, pH, phosphates, chlorates, free chlorine, sodium, total organic carbon, bromodichloromethane, dibromochloroethane and ∑TMHs, for which $p < 0.05$. These variables separated water samples that contain *Hp* (1) DNA and do not contain *Hp* (0) DNA. LDA was performed based on the same selected variables in the next step. LDA analysis correctly differentiated 184 (86.0%) of all samples

Table 5. List of the parameters that were examined in water samples from different WTP, units and p-values for the tests that examined statistically significant differences for the samples with *Hp* (1) DNA and the samples without *Hp* (0) DNA. Asterisk means statistical significance at $p < 0.05$. Double asterisks mean that it was not possible to perform the test due to the lack of differentiation of the variable's values.

	Parameter	Unit	WTP			
			Bielany (Sanka river)	Rudawa	Dlubnia	Raba Rudawa & Raba
			(p-value)			
1	total number of microorganisms at 22ºC after 72 h	jtk/mL	0.550	0.725	0.922	0.355
2	color	mg/L Pt	0.331	0.007*	0.627	0.039*
3	pH	–	0.200	0.547	0.205	0.000*
4	conductivity at 25°C	µS/cm	0.060	0.152	0.770	0.206
5	chlorides	mg/L	0.129	0.337	0.018*	0.059
6	nitrites	mg/L	0.437	0.017*	0.683	0.157
7	nitrates	mg/L	0.308	0.468	0.443	0.084
8	phosphates	mg/L	0.261	0.191	0.077*	0.009*
9	chlorates	mg/L	0.230	0.312	0.362	0.011*
10	sulphates	mg/L	0.884	0.201	0.014*	0.706
11	free chlorine	mg/L	0.107	0.877	0.162	0.016*
12	total iron	mg/L	0.121	0.929	0.273	0.195
13	aluminum	mg/L	0.146	0.345	0.731	0.231
14	sodium	mg/L	0.105	0.608	0.475	0.000*
15	ammonium ion	mg/L	0.817	0.576	0.611	0.408
16	potassium	mg/L	0.377	0.851	0.601	0.438
17	magnesium	mg/L	0.014*	0.862	0.817	0.542
18	calcium	mg/L	0.220	0.075	0.888	0.319
19	fluorides	mg/L	0.489	0.184	0.334	0.784
20	chlorites	mg/L	**	0.178	0.824	0.112
21	sum of chlorates and chlorites	mg/L	**	0.331	0.712	0.062
22	organic carbon OWO	mg/L	0.127	0.002*	0.266	0.026*
23	trichloromethane	µg/L	0.003*	0.416	0.835	0.067
24	bromodichloromethane	µg/L	0.001*	0.244	0.749	0.000*
25	dibromochloroethane	µg/L	0.511	0.848	0.275	0.000*
26	∑TMHs	µg/L	0.727	0.993	0.888	0.005*

Table 6. LDA results for the water samples collected from WTP Bielany (Sanka river).

	***Hp* (0) DNA detected**	***Hp* (1) DNA detected**
***Hp* (0) DNA measured**	15 71.4%	6 28.6%
***Hp* (1) DNA measured**	1 6.7%	14 93.3%

from WTP Raba and WTP Rudawa & WTP Raba, including 86 (84.3%) of the correct samples labeled *Hp* (0) DNA and 98 (87.5%) the correct samples labeled *Hp* (1) DNA (*Table 9*).

It was concluded that, based on the selected variables, LDA correctly recognizes *Hp* (1) DNA and *Hp* (0) DNA with the accuracy above 86%.

Analysis of Hp DNA presence in seasons of the years 2012 – 2016

We also analyzed the presence of *Hp* DNA in the water samples collected by season (*Table 10*). We found the highest number of water samples containing *Hp* DNA (*glmM*) in the summer. Among the 79 samples tested in summer, 73 (92.41%) water samples contained *Hp* DNA. We also detected a greater number of samples expressing *glmM* gene in autumn (58.73%) while the least in spring (28.74%).

We checked whether all 26 variables allow to distinguish the seasons of the year when the samples were taken (*Fig. 9*). Using PC1, PC2 and PC3, it was not possible to realize our goal.

The variability of individual principal components (PC) and the projection of points (water samples) using PC1-PC3 in seasons is presented in *Fig. 9*. Dots of individual colors represent the water samples collected during seasons in the year.

Our next goal was to investigate in each season of the year separately, which of the variables differentiate samples containing *Hp* (1) DNA from those not containing *Hp* (0) DNA. The p-values are given in *Table 11*. A significant variables are marked with an asterisk ($p < 0.05$). Then we have employed PCA analysis for each season considering only these differentiating variables. The water samples collected in spring and summer were omitted because there were not enough differentiating variables to perform PCA and LDA analyses. There are only two variables for spring and only one variable for summer ($p < 0.05$) showing difference in the water samples. The calculations were continued for water samples taken in the autumn and winter.

Fig. 10 and *Fig. 11* show that by considering of the selected variables in autumn and winter (marked with asterisk in *Table 7*), it is possible to distinguish the samples with *Hp* (1) DNA from those without *Hp* (0) DNA.

As shown in *Fig. 10* total of 9 variables have been considered for analysis, namely, chlorides, total iron, sodium, potassium, fluorides, organic carbon OWO, trichloromethane, bromodichloromethane and ∑TMHs, for which $p < 0.05$. These variables separated water samples into those that contain *Hp* (1) DNA from those not containing *Hp* (0) DNA. The LDA was performed using the selected variables in the next step. LDA correctly differentiated 104 (82.5%) of all samples collected in autumn. Based on the carried out analysis, we concluded that in autumn 37 (71.2%) of the samples were correctly-labeled *Hp* (0) DNA and 67 (90.5%) of the correctly-labeled *Hp* (1) DNA samples (*Table 12*).

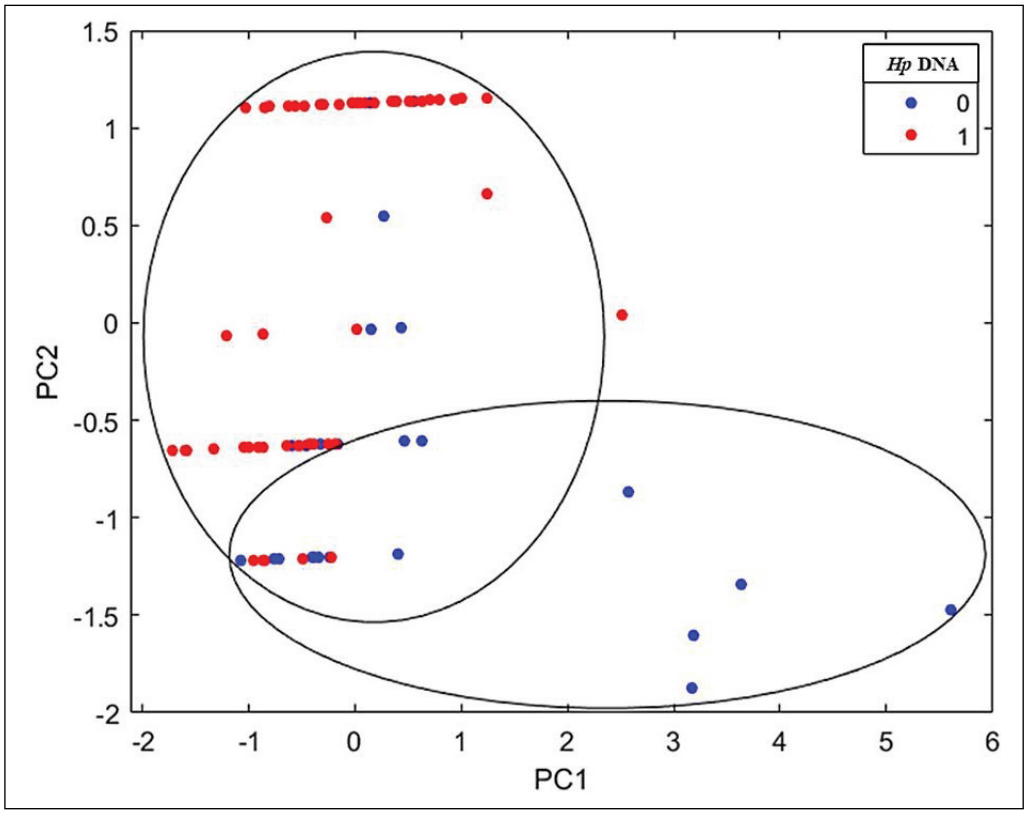

Fig. 6. Projection of points representing the water samples from WTP Rudawa on the PC1/PC2 plane. The sets of water samples are marked with two ellipses.

Table 7. LDA results for the water samples collected from WTP Rudawa.

	***Hp* (0) DNA detected**	***Hp* (1) DNA detected**
***Hp* (0) DNA measured**	10 31.2%	22 68.8%
***Hp* (1) DNA measured**	3 5.3%	54 94.7%

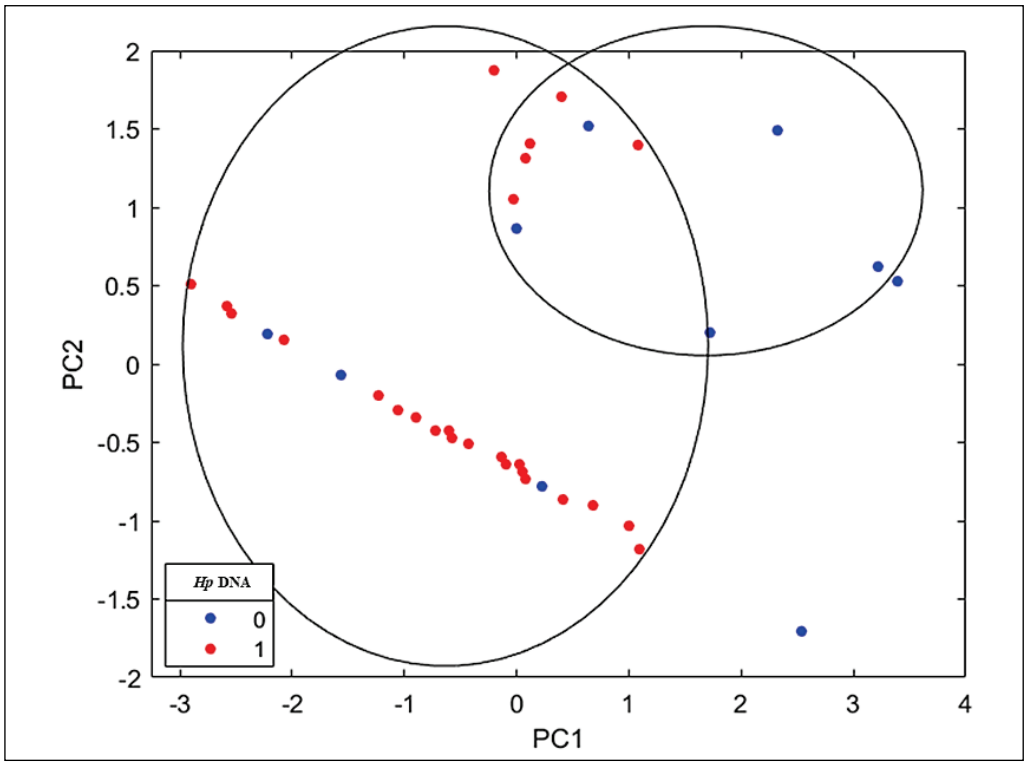

Fig. 7. Projection of points representing the water samples from WTP Dlubnia on the PC1/PC2 plane. The sets of water samples are marked with two ellipses.

Table 8. LDA results for the water samples collected from WTP Dlubnia.

	***Hp* (0) DNA detected**	***Hp* (1) DNA detected**
***Hp* (0) DNA measured**	5 41.7%	7 58.3%
***Hp* (1) DNA measured**	3 10.7%	25 89.3%

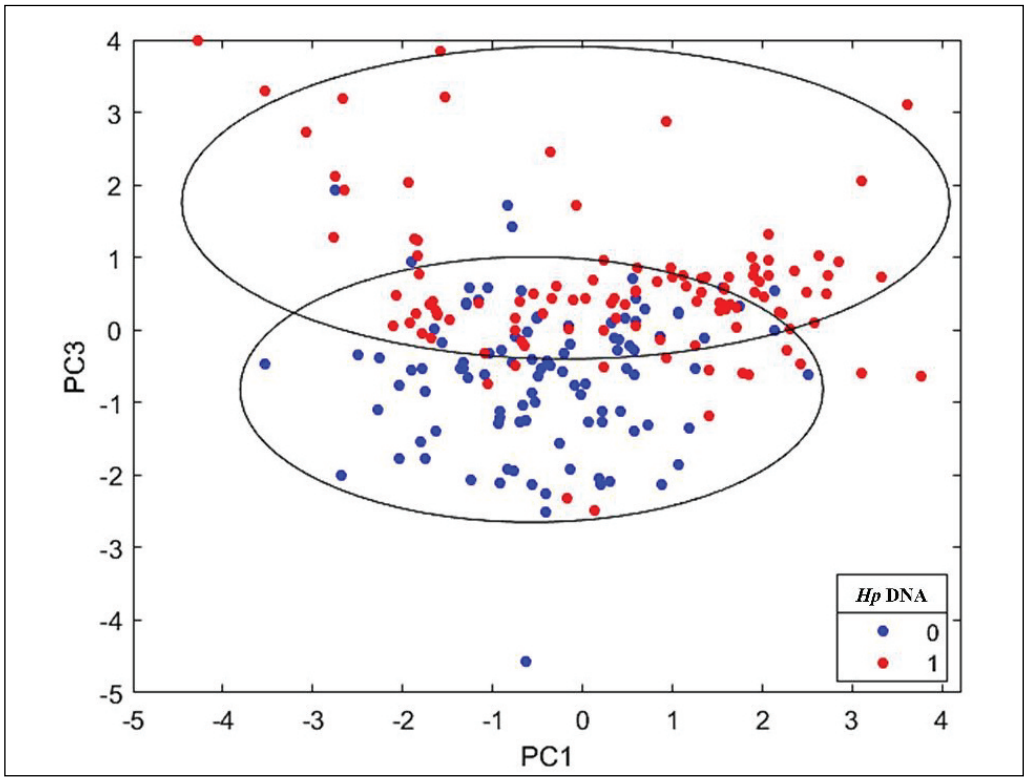

Fig. 8. Projection of points representing the water samples from WTP Raba and WTP Rudawa & WTP Raba on the PC1/PC3 plane. The sets of water samples are marked with two ellipses.

Table 9. LDA results for the water samples collected from WTP Raba and WTP Rudawa & WTP Raba.

	***Hp* (0) DNA detected**	***Hp* (1) DNA detected**
***Hp* (0) DNA measured**	86 84.3%	16 15.7%
***Hp* (1) DNA measured**	14 12.5%	98 87.5%

Table 10. Number of water samples and frequency of *Hp* DNA (*glmM*) occurrence in seasons.

	Season	**Number of water samples**	***Hp (1)* DNA**	***Hp* (1) DNA %**	***Hp* (0) DNA**	***Hp* (0) DNA %**
1	**Spring**	87	25	28.74	62	71.26
2	**Summer**	79	73	92.41	6	7.59
3	**Autumn**	126	74	58.73	52	41.27
4	**Winter**	87	40	45.98	47	54.02

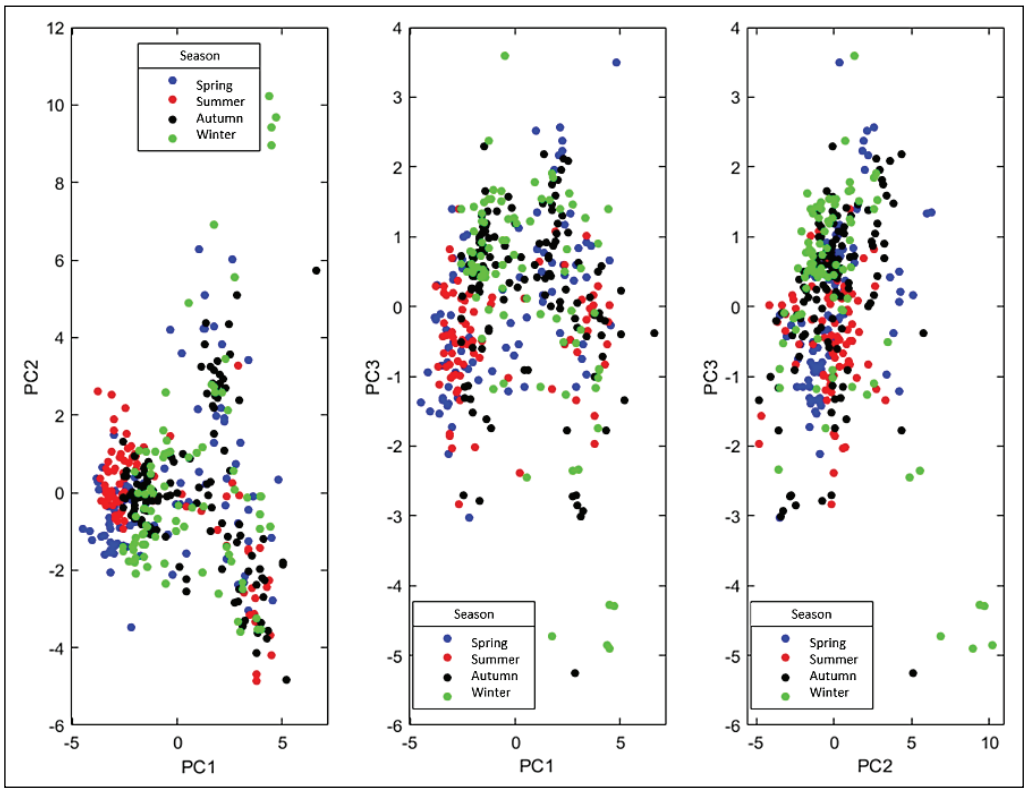

Fig. 9. Projection of the points representing all water samples and all variables in individual seasons of the year on the PC1/PC2, PC1/PC3 and PC2/PC3 planes.

It was concluded that, LDA correctly recognized *Hp* (1) DNA and *Hp* (0) DNA with the accuracy above 82%.

The choice of 17 variables for further analysis including the following parameters: pH, conductivity at 25ºC, chlorides, nitrates, chlorates, phosphates, sulfates, sodium, ammonium ion, potassium, magnesium, calcium, chlorites, sum of chlorates and chlorites, trichloromethane, bromodichloromethane and dibromochloroethane is presented in *Fig. 11* ($p < 0.05$). These variables separate water samples that contain *Hp* (1) DNA from those which do not contain *Hp* (0) DNA. The LDA was performed using on the same selected variables in the next step. LDA has correctly differentiated 83 (95.4%) of all samples collected in winter, including 46 (97.9%) of the correct samples labeled *Hp* (0) DNA and 37 (92.5%) of the samples labeled correctly *Hp* (1) DNA (*Table 13*).

It was concluded, that, LDA correctly recognizes *Hp* (1) DNA and *Hp* (0) DNA with the accuracy above 95%.

DISCUSSION

Despite the several epidemiological studies supporting the hypothesis that *Hp* is a waterborne pathogen, the real contribution of drinking water to the spread of the pathogen remains a matter of debate (3-16). Evaluation of chemical and microbial contaminants is essential for the drinking water safety, however, *Hp* is not considered in routine microbiological analysis of drinking water in many countries world-wide including Poland.

The presence of *Hp* DNA in drinking water systems has been reported in the Columbia, Peru, Pakistan, England, Iran, Spain, Egypt and Japan (4, 5, 7, 13, 14, 49, 53, 54). Also in our work, this aspect of *Hp* water contamination comes to the fore because we attempted to determine the prevalence of *Hp* in tap water samples. We used molecular PCR-based method using primers designed from conserved regions of the *Hp* urease C gene (*glmM*

Table 11. The list of parameters that were examined in the water samples, units and p-values for the tests which revealed statistically significant differences for the samples with and without (*Hp* (1) DNA, *Hp* (0), respectively) DNA. Asterisk means $p < 0.05$.

	Parameter	Unit	Season			
			Spring	Summer	Autumn	Winter
			p-value			
1	total number of microorganisms at 22ºC after 72 h	jtk/1mL	0.233	0.588	0.942	0.1587
2	colour	mg/L Pt	0.141	0.174	0.647	0.2892
3	pH		0.580	0.431	0.929	0.0000*
4	conductivity at 25ºC	µS/cm	0.057	0.176	0.776	0.0000*
5	chlorides	mg/L	0.123	0.126	0.028*	0.0000*
6	nitrites	mg/L	0.008*	0.519	0.237	1.0000
7	nitrates	mg/L	0.157	0.071	0.172	0.0005*
8	phosphates	mg/L	0.127	0.524	0.826	0.0145*
9	chlorates	mg/L	0.585	0.306	0.365	0.0000*
10	sulphates	mg/L	0.219	0.298	0.074	0.0000*
11	free chlorine	mg/L	0.642	0.575	0.230	0.0746
12	total iron	mg/L	0.648	0.276	0.035*	0.6071
13	aluminum	mg/L	0.154	0.281	0.061	0.2754
14	sodium	mg/L	0.018*	0.136	0.000*	0.0022*
15	ammonium ion	mg/L	0.180	0.000*	0.234	0.0228*
16	potassium	mg/L	0.407	0.324	0.000*	0.0148*
17	magnesium	mg/L	0.282	0.198	0.345	0.0000*
18	calcium	mg/L	0.160	0.139	0.962	0.0000*
19	fluorides	mg/L	0.485	0.726	0.005*	0.1180
20	chlorites	mg/L	0.103	0.907	0.675	0.0018*
21	sum of chlorates and chlorites	mg/L	0.217	0.775	0.429	0.0007*
22	organic carbon OWO	mg/L	0.477	0.806	0.009*	0.6905
23	trichloromethane	µg/L	0.166	0.575	0.001*	0.0000*
24	bromodichloromethane	µg/L	0.766	0.305	0.005*	0.0198*
25	dibromochloroethane	µg/L	0.257	0.586	0.079	0.0106*
26	∑TMHs	µg/L	0.207	0.526	0.005*	0.8740

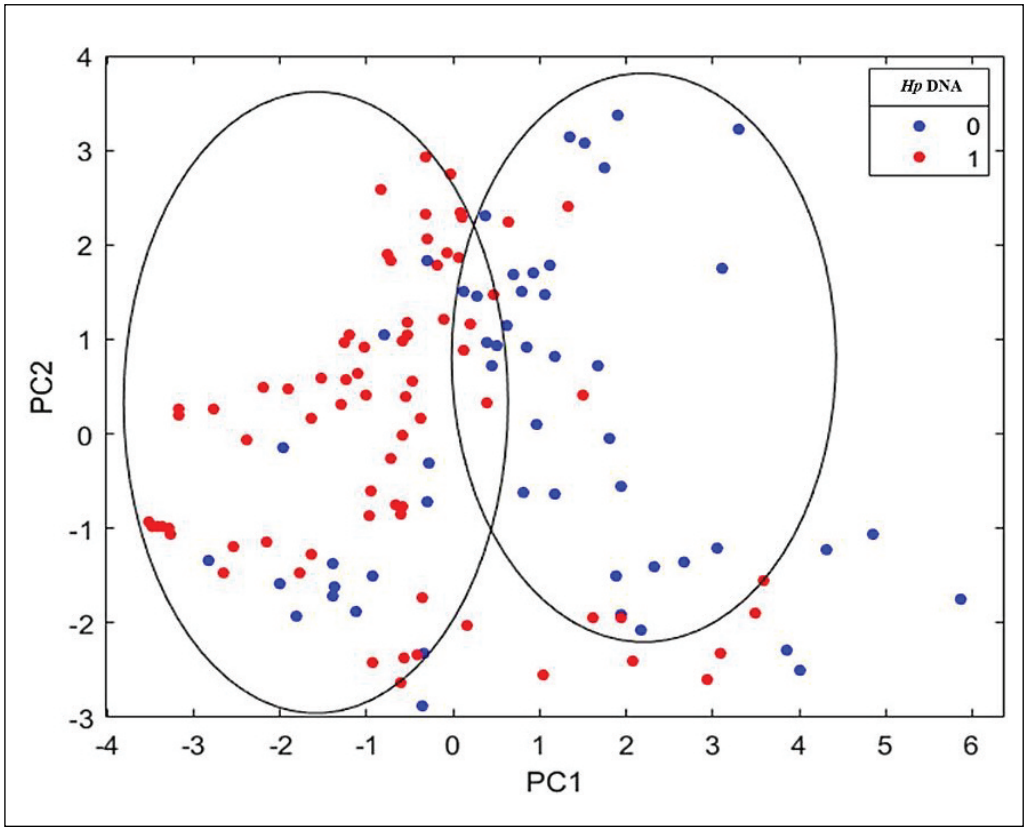

Fig. 10. Projection of points representing the water samples collected in autumn on the PC1/PC2 plane. The sets of water samples marked with two ellipses.

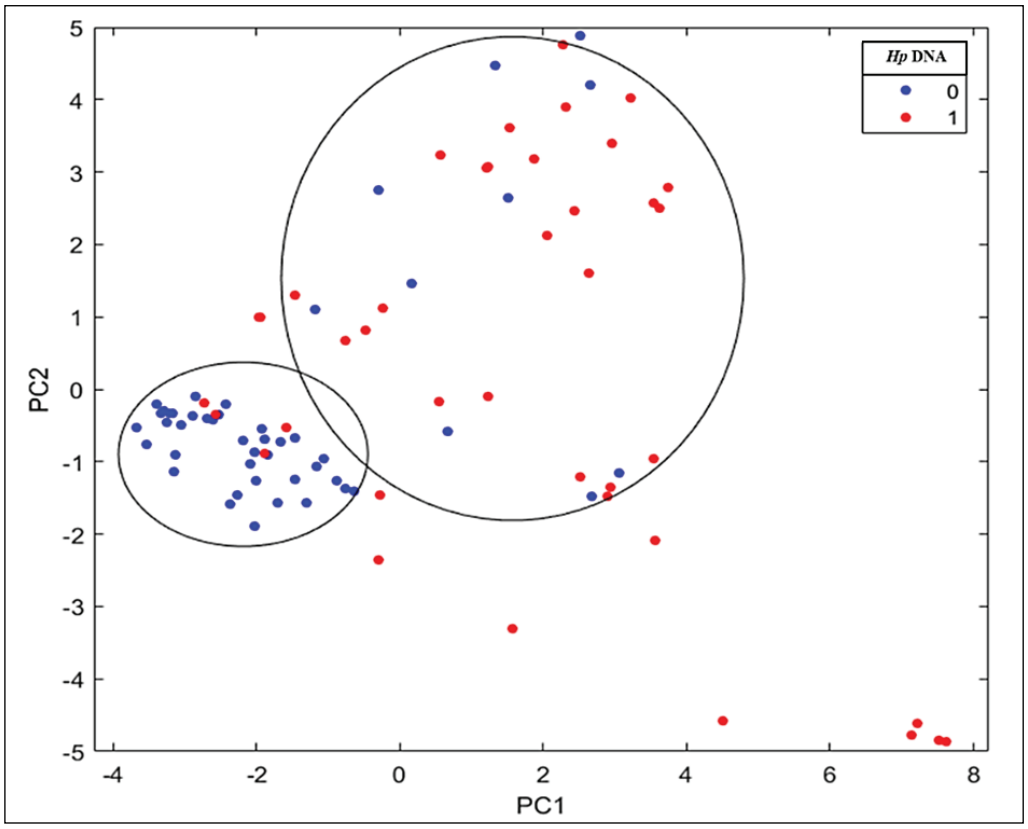

Fig. 11. Projection of points representing the water samples collected in winter on the PC1/PC2 plane. The sets of water samples were marked with two ellipses.

Table 12. LDA results for the water samples collected in autumn.

	***Hp* (0) DNA detected**	***Hp* (1) DNA detected**
***Hp* (0) DNA measured**	37 71.2%	15 28.8%
***Hp* (1) DNA measured**	7 9.5%	67 90.5%

Table 13. LDA results for the water samples collected in winter.

	***Hp* (0) DNA detected**	***Hp* (1) DNA detected**
***Hp* (0) DNA measured**	46 97.9%	1 2.1%
***Hp* (1) DNA measured**	3 7.5%	37 92.5%

gene). This gene acts as a housekeeping gene and is essential for *Hp* growth and survival (55). In addition, *glmM* sequences are relatively well conserved and present in all *Hp* isolates. The *glmM* gene has a high degree of sensitivity and specificity (55).

We found that *Hp* DNA was present in 55.96% (212/379) tap water samples collected from different places of the city of Cracow. To our best knowledge this is the first sampling study of drinking water contamination with *Hp* in Poland. Our findings revealed that *Hp* DNA detection was quite frequent in comparison with studies published by others, on the tap water samples from Bogota 38.7%, Lima 20.3 %, Lahore (Pakistan) 40.0% and Kermanshah (Iran) 36% (4, 5, 49, 56). Herein, we provide the evidence on the relationship between presence of *Hp* DNA and ionic and the organic composition of microelements that was observed in drinking water of Cracow.

The sources of raw water for individual water treatment plants for Cracow are rivers: Sanka for WTP Bielany, Dlubnia for WTP Dlubnia, Rudawa for WTP Rudawa, Raba for WTP Raba (*Fig. 1*). Water samples were collected from 52 points which were located within the area supplied by these WTPs (*Table 1*). Previous studies on the relationship of physicochemical parameters to the presence of *Hp* in water have been conducted, however, the interpretation of these findings are limited due to a small number of these parameters assessed (4, 5, 49, 57, 58).

West *et al.* (57) have documented the prolonged *Hp* survival in water, saline, and various buffers at room temperature over a range of physical variables. They found that survival rates among strains were varied. Interestingly, *Hp* can survive in distilled water and the concentration of 0 – 6 M sodium chloride (the approximate salinity of seawater) for a significant period of time. It was evidenced that *Hp* can survive in natural aquatic environments with pH ranging from 3.0 to10.0 (57).

Herein, we have examined 26 parameters in collected water samples, namely: total number of microorganisms at 22ºC, color, pH, conductivity at 25ºC, chlorides, nitrites, nitrates, phosphates,

chlorates, sulphates, free chlorine, total iron, aluminum, sodium, ammonium ion, potassium, magnesium, calcium, fluorides, chlorites, sum of chlorates and chlorites, total organic carbon, trichloromethane, bromodichloromethane, dibromochloroethane and ∑TMHs. In contrast, the available evidence based medicine accumulated so far, has indicated the determination of less number of parameters pH, temperature, free chlorine, turbidity and conductivity (4, 5, 49, 56).

The PCA method used allowed us to isolate clusters of the water samples collected from WTPs. The result of this analysis, based on all 26 variables, confirmed the similarity of the samples belonging to the particular WTP. Therefore, the next steps of our work were related to individual WTPs. The PC (principal components) extracted in the PCA method, made possible to distinguish the objects (water samples) containing *Hp* (1) DNA from those do not containing *Hp* (0) DNA in each WTP. In WTP Bielany (Sanka river) the following 3 variables have been analyzed, namely: magnesium, trichloromethane and bromodichloromethane while another 3 variables, namely: color, nitrites and total organic carbon were considered for WTP Rudawa. In WTP Dlubnia also 3 variables were considered, namely: chlorites, phosphates and sulfates while in WTP Raba and WTP Rudawa & WTP Raba 10 variables have been analyzed including the color, pH, phosphates, chlorates, free chlorine, sodium, total organic carbon, bromodichloromethane, dibromochloroethane and ∑TMHs. These variables allowed water samples to be separated to either containing *Hp* (1) DNA and not containing *Hp* (0) DNA ($p < 0.05$).

The next step was to apply the LDA method, which allowed recognition of *Hp* (1) DNA positive samples using only selected variables in each particular WTP. It also allowed the recognition of negative samples in the WTP Raba and the WTP Rudawa & WTP Raba using selected variables. LDA correctly identified positive *Hp* (1) DNA water samples and negative *Hp* (0) DNA water samples in WTP Raba and WTP Rudawa & WTP Raba. In this analysis, 86% of the test sites were included in the appropriate group.

Interestingly, Amirhooshang *et al.* (49) showed that the average concentration of free chlorine in tap water was significantly lower than that recommended by the World Health Organization (WHO) standards. They found a statistically significant relationship between the DNA of *Hp* positive samples and free chlorine concentration and temperature using PCR. The *Hp* positive water samples had significantly lower free chlorine levels and lower temperature (49).

Santiago *et al.* (58) have employed the culture and molecular (qPCR, PMA-qPCR, DVC-FISH, FISH) methods to detect the presence of live *Hp* cells in twenty four drinking water samples collected in eastern Spain. They analyzed fifteen public water fountains and six domestic taps, all of them belonging to the public drinking water distribution system and supplied by the same water treatment plant (58). The presence of viable *Hp* cells was identified in only six public fountains but none of viable *Hp* microorganism in domestic tap water was detected. All samples were taken from the final distribution points (tap or public fountain) (58). The residual chlorine in the analyzed samples averaged between 0.5 – 0.9 mg/L, considered as the normal levels within range in drinking water (25). No relationship was observed between the presence of alive *Hp* cells, and location of water sampling as well as between presence of alive *Hp* cells and chlorine levels (58).

Interestingly in our present study, the free chlorine in WTP Raba and WTP Rudawa & WTP Raba was within the proper range limit of which allowed water samples to be separated from those containing *Hp* (1) DNA from those not containing *Hp* (0) DNA. Vesga *et al.* (4) failed to demonstrate the statistically significant relationships between the physicochemical parameters such as turbidity, conductivity, pH, residual free chlorine and the presence or absence of *Hp* in the raw and drinking water samples. They have suggested (4) that the obtained differences in the values of these physicochemical parameters cannot be taken into account to draw definitive conclusions regarding the presence or absence of *Hp* in raw or drinking water. The results of our study are in variance from those obtained by Vesga *et al.* (4) because based on the selected physicochemical parameters, both PCA and LDA methods were correctly able to recognize water samples containing *Hp* (1) DNA from those not containing *Hp* (0) DNA. The relationship between physicochemical parameters and the presence of *Hp* in drinking water samples was further explored by Boehnke *et al.* (5) who found that the increased temperature was associated with a lower likelihood of the presence of *Hp* and that the increased pH was associated with a higher quantity of *Hp* (5). Our analyses also showed that pH can be a parameter that can differentiate the water samples containing *Hp* (1) DNA from those not containing *Hp* (0) DNA.

Hortelano *et al.* (59) have described culture media which were designed and tested for the isolation of *Hp* from environmental samples. Due to the nature and living conditions of this microorganism, the determination of selective agents that inhibit large number of contaminant microbiota, while allowing successful growth of *Hp* is extremely difficult. In their study no correlation between successful cultivation and inorganic compounds, such as sodium chloride (5 g/L), sodium bisulfite (0.1 g/L), ferric nitrate (0.02 g/L) was found (59). On the other hand, in our study, sodium in WTP Dlubnia and chlorides in WTP Raba and WTP Rudawa & WTP Raba were among the selected variables which allowed water samples to be separated to those containing *Hp* (1) DNA and not containing *Hp* (0) DNA.

Determination of the relationship between season and the frequency of *Hp* DNA in water and plant foods (vegetable and salad samples) has been undertaken in studies conducted in Colombia, Peru, Iran, Greece (4, 5, 15, 41, 42). In our study, the highest number of samples containing *Hp* DNA during the summer has been detected. During this time of year, we detected 92.41% of water samples which showed glmM expression (*Table 10*). Ranjbar *et al.* (15) reported a similar seasonal distribution for the *Hp* strains because the highest prevalence of *Hp* (4.54 %) was achived in the bottled mineral water samples which were collected in the summer season. They found significant statistical difference for the prevalence of *Hp* between warm and cold seasons of the year ($p < 0.05$) (15). The bottled mineral water samples from July had the highest levels (50%) of *Hp*-contamination (15). Also, Yahaghi *et al.* (41) identified marked seasonality in the incidence of *Hp* isolated from vegetable and salad samples but *Hp* isolates had the highest incidence (71, 18%) in spring season. In contrast, we were unable to detect the higher incidence of *Hp*-contaminated water samples in the spring.

Interestingly, testing of drinking water samples in another studies failed to show associations between the presence or absence of *Hp* and the seasons (4, 5). Similarly, Tirodimos *et al.* who also tested water samples taken from the Aliakmon River, in Greece failed to observe both, the seasonal variation and the associated correlation between particular season and the occurrence of *Hp* (42).

In our present study the PCA method did not allow to isolate clusters of water samples collected in seasons. The result of this analysis, concerning all 26 variables, failed to confirm similarity of the samples belonging to particular seasons. PC (principal components) extracted in PCA method, made it possible to distinguish the objects (water samples) containing *Hp* (1) DNA from those do not containing *Hp* (0) DNA only in autumn and winter. In autumn, 9 selected variables were analyzed including: chlorides, total iron, sodium, potassium, fluorides, organic carbon

OWO, trichloromethane, bromodichloromethane and ∑TMHs. In winter there were 17 selected variables analyzed, namely: pH, conductivity at 25ºC, chlorides, nitrates, chlorates, phosphates, sulfates, sodium, ammonium ion, potassium, magnesium, calcium, chlorites, sum of chlorates and chlorites, trichloromethane, bromodichloromethane and dibromochloroethane. These variables separated water samples into those that contain *Hp* DNA from those not containing *Hp* DNA ($p < 0.05$).

In autumn and winter, using LDA method we were able to detect correctly positive (*Hp* (1) DNA) and negative (*Hp* (0) DNA) water samples. LDA correctly differentiated 82.5% and 95.4% of all samples collected in autumn and winter, respectively. Additionally, LDA correctly identified 92.5% (37/40) of positive (*Hp* (1) DNA) water samples and 97.9% (46/47) of negative (*Hp* (0) DNA) water samples collected in winter time. Also in winter, based on seventeen of the twenty-six variables, we were able to distinguish water samples containing *Hp* (1) DNA from those not containing *Hp* (0) DNA. This clearly indicates that using this method we can distinguish water samples containing *Hp* DNA with high probability from those not containing *Hp* DNA.

The detection of such a large number of *Hp* DNA - containing water samples during the summer (92.41%) resulted in almost all samples belonging to a single set. Additionally, there was only one variable ($p < 0.05$) recorded for summer that distinguished *Hp* DNA containing samples from non *Hp* DNA containing water samples and therefore we did not conduct PCA and LDA analysis (*Table 10*). It would have been pointless to conduct the analysis on unbalanced collections having among 79 samples tested, as many as 73 were positive for *Hp* DNA. Spring was also omitted because there were only two variables whose values differed within water samples.

To date, no studies detecting *Hp* DNA and evaluating the genotypic profile of *Hp* strains have been conducted in collected water samples from the city Cracow. Infected patients with strains expressing the *cagA* gene were more exposed to develop gastric cancer than those infected with *cagA*-negative strains (37). In our study among 212 *glmM* positive samples, *cagA* gene was detected in 145 (68.40%) water samples (*Table 4*). Our results were similar to the results obtained by Ranjbar *et al.* because they showed that 62.5% of DNA *Hp* identified in bottled mineral water samples had the *cagA* genotype (15). In another study conducted in Mexico, the *cagA* gene expression was confirmed in 42% of raw water samples collected from different water systems (44).

According to our study, the consuming *Hp*-contaminated tap water may lead to gastrointestinal disorders caused by the infection or presence of virulent strains of *Hp*. Previous studies revealed that the strain *Hp* ATTC 43504 positive for *cagA* gene and protein *CagA* may be involved in the molecular mechanisms of activation of fibroblasts which can switch gastric epithelium microevolution towards cancer stem cell-related differentiation program that can potentially initiate gastric carcinogenesis (60-64). In fact, the multicenter study in Poland provided analysis on almost seven thousand individuals assessed by the presence of antibodies in IgG class against *Hp* in serum (65). In children *cagA s1m1* was observed more frequently than in adults (34% versus 23.1%; $p = 0.02$), predicting more severe consequences of the infection in adulthood (65). Therefore, future studies should focus on the identification of the genotype of *Hp* strains expressing the *cagA* gene that may be present in drinking water exerting a deleterious effect on the health of human body.

Although *Hp* has been proposed by USEPA as a new drinking water contaminant, only few studies presented the standardized procedures for its detection in water. There are no regulations in Poland or other countries that allow for routine and standardized testing of drinking water samples.

The ionic and organic composition of micronutrients in water can influence the presence of *Hp*. Thus, as shown in our present study the determination of selected micronutrients indicates and sometimes may predict the presence of *Hp* in drinking water. Winter is the time of year when, based on selected variables, we could most accurately distinguish samples containing *Hp* DNA from those that do not contain *Hp* DNA. We therefore, propose that routine testing for detection of the *Hp* DNA in water should be conducted during this season of the year. Moreover, we propose to include the determination of selected micronutrients in drinking water samples into routine procedures used in WTPs.

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Conflict of interests: None declared.

REFERENCES

1. Percival SL, Thomas JG. Transmission of Helicobacter pylori and the role of water and biofilms. *J Water Health* 2009; 7: 469-477. doi: 10.2166/wh.2009.070
2. Kotilea K, Bontems P, Touati E. Epidemiology, diagnosis and risk factors of Helicobacter pylori infection. *Adv Exp Med Biol* 2019; 1149: 17-33.
3. Moreno Y, Ferrus MA. Specific detection of cultivable Helicobacter pylori cells from wastewater treatment plants. *Helicobacter* 2012; 7: 327-332.
4. Vesga FJ, Moreno Y, Ferrus MA, Ledesma-Gaitan LM, Campos C, Trespalacios AA. Correlation among fecal indicator bacteria and physicochemical parameters with the presence of Helicobacter pylori DNA in raw and drinking water from Bogota, Colombia. *Helicobacter* 2019; 24: e12582. 10.1111/hel.12582
5. Boehnke KF, Brewster RK, Sanchez BN, *et al.* An assessment of drinking water contamination with Helicobacter pylori in Lima, Peru. *Helicobacter* 2018; 23: e12462. doi: 10.1111/hel.12462
6. Duarte N, Salazar V, Casanova G, Suarez P, Fernandez-Delgado M. Occurrence of Helicobacter spp. and fecal bacterial contamination in high-altitude aquatic environments from the Andes. *Bull Environ Contam Toxicol* 2021: 107: 433-440.
7. Moreno-Mesonero L, Moreno Y, Alonso JL, Ferrus MA. Detection of viable Helicobacter pylori inside free-living amoebae in wastewater and drinking water samples from Eastern Spain. *Environ Microbiol* 2017; 19: 4103-4112.
8. Dey R, Rieger A, Banting G, Ashbolt NJ. Role of amoebae for survival and recovery of 'non-culturable' Helicobacter pylori cells in aquatic environments. *FEMS Microbiol Ecol* 2020; 96: fiaa182. doi: 10.1093/femsec/fiaa182
9. Vesga FJ, Moreno Y, Ferrus MA, Campos C, Trespalacios AA. Detection of Helicobacter pylori in drinking water treatment plants in Bogota, Colombia, using cultural and molecular techniques. *Int J Hyg Environ Health* 2018; 221: 595-601.
10. Assadi MM, Chamanrokh P, Whitehouse CA, *et al.* Methods for detecting the environmental coccoid form of Helicobacter pylori. *Front Public Health* 2015; 3: 147. doi: 10.3389/fpubh.2015.00147
11. Chamanrokh P, Shahhosseiny MH; Assadi MM, *et al.* Three tests used to identify non-culturable form of Helicobacter pylori in water samples. *Jundishapur J Microbiol* 2015; 8: e16811. doi: 10.5812/jjm.8(4)2015.16811
12. Dinda V, Kimang A. Co-occurrence of Helicobacter pylori with faecal bacteria in Nairobi river basin: public health

implications. *African Health Sciences* 2016; 16: 177-182.
13. El-Sharouny E, El-Shazli H, Olama Z. Detection of Helicobacter pylori DNA in some egyptian water systems and its incidence of transmission to individuals. *Iran J Public Health* 2015; 44: 203-210.
14. Horiuchi S, Nakano R, Nakano A, *et al.* Prevalence of Helicobacter pylori among residents and their environments in the Nara prefecture, *Japan J Infect Public Health* 2021; 14: 271-275.
15. Ranjbar R, Khamesipour F, Jonaidi-Jafari N, Rahimi E. Helicobacter pylori in bottled mineral water: genotyping and antimicrobial resistance properties. *BMC Microbiol* 2016; 16: 40. doi: 10.1186/s12866-016-0647-1
16. Bahrami AR, Rahimi E, Ghasemian Safaei H. Detection of Helicobacter pylori in city water, dental units' water, and bottled mineral water in Isfahan, Iran. *Scientific World Journal* 2013; 2013: 280510. doi: 10.1155/2013/280510
17. United States Environmental Protection Agency. Contaminant Candidate List 5 (CCL 5). Announcement of the Draft CCL 5 2021; [https://www.epa.gov/ccl/draft-ccl-5-microbial-contaminants]
18. WHO Publishes List of Bacteria for which New Antibiotics are Urgently Needed [https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed]
19. Quaglia NC, Dambrosio A. Helicobacter pylori: a foodborne pathogen? *World J Gastroenterol* 2018; 24: 3472-3487.
20. Percival SL, Suleman L. Biofilms and Helicobacter pylori: dissemination and persistence within the environment and host. *World J Gastroint Pathophy* 2014; 5: 122-132.
21. Azevedo NF, Almeida C, Cerqueira L, Dias S, Keevil CW, Vieira MJ. Coccoid form of Helicobacter pylori as a morphological manifestation of cell adaptation to the environment. *Appl Environ Microbiol* 2007; 731: 3423-3427.
22. Li L, Mendis N, Trigui H, Oliver JD, Faucher SP. The importance of the viable but non-culturable state in human bacterial pathogens. *Front Microbiol* 2014; 5: 258. doi: 10.3389/fmicb.2014.00258
23. Mazari-Hiriart M, Lopez-Vidal Y, Ponce de Leon S, Castillo-Rojas G, Hernandez-Eugenio C, Rojo F. Bacteria and disinfection by products in water from southern Mexico City. *Arch Environ Health* 2003; 58: 233-237.
24. Moreno Y, Piqueres P, Alonso JL, Jimenez A, Gonzalez A, Ferrus MA. Survival and viability of Helicobacter pylori after inoculation into chlorinated drinking water. *Water Res* 2007; 41: 3490-3496.
25. Baker KH, Hegarty JP, Redmond B, Reed NA, Herson DS. Effect of oxidizing disinfectants (chlorine monochloramine and ozone) on Helicobacter pylori. *Appl Environ Microbiol* 2002; 68: 981-984.
26. Orta de Velasquez MT, Yanez Noguez I, Casasola Rodriguez B, Roman Roman PI. Effects of ozone and chlorine disinfection on VBNC Helicobacter pylori by molecular techniques and FESEM images. *Environ Technol* 2017; 38: 744-753.
27. De Reuse H, Labigne A, Mengin-Lecreulx D. The Helicobacter pylori ureC gene codes for a phosphoglucosamine mutase. *J Bacteriol* 1997; 179: 3488-3493.
28. Lu JJ, Perng CL, Shyu RY, *et al.* Comparison of five PCR methods for detection of Helicobacter pylori DNA in gastric tissues. *J Clin Microbiol* 1999; 37: 772-774.
29. Bickley J, Owen RJ, Fraser AG, Pounder RE. Evaluation of the polymerase chain reaction for detecting the urease C gene of Helicobacter pylori in gastric biopsy samples and dental plaque. *J Med Microbiol* 1993; 39: 338-344.
30. Cellini L, Del Vecchio A, Di Candia M, Di Campli E, Favaro M, Donelli G . Detection of free and plankton-associated Helicobacter pylori in seawater. *J Appl Microbiol* 2004; 97: 285-292.
31. Shahamat M, Alavi M, Watts JE, *et al.* Development of two PCR-based techniques for detecting helical and coccoid forms of Helicobacter pylori. *J Clin Microbiol* 2004; 42: 3613-3619.
32. Shetty V, Lingadakai R, Pai GC, Ballal M. Profile of Helicobacter pylori cagA & vacA genotypes and its association with the spectrum of gastroduodenal disease. *Indian J Med* 2021; 22: S0255-0857(21)04125-6. doi: 10.1016/j.ijmmb.2021.06.001
33. Nilsson C, Sillen A, Eriksson L, *et al.* Correlation between cag pathogenicity island composition and Helicobacter pylori-associated gastroduodenal disease. *Infect Immun* 2003; 71: 6573-6581.
34. Covacci A, Telford JL, Del Giudice G, Parsonnet J, Rappuoli R. Helicobacter pylori virulence and genetic geography. *Science* 1999; 284: 1328-1333.
35. Lima VP, Silva-Fernandes IJ de L, Alves MK, Rabenhorst SH. Prevalence of Helicobacter pylori genotypes (vacA, cagA, cagE and virB11) in gastric cancer in Brazilian's patients: an association with histopathological parameters. *Cancer Epidemiol* 2011; 35: e32-e37. doi: 10.1016/j.canep.2011.02.017
36. Nguyen TL, Uchida T, Tsukamoto Y, *et al.* Helicobacter pylori infection and gastroduodenal diseases in Vietnam: a cross-sectional, hospital-based study. *BMC Gastroenterol* 2010; 10: 114. doi: 10.1186/1471-230X-10-114
37. Takahashi-Kanemitsu A, Knight CT, Hatakeyama M. Molecular anatomy and pathogenic actions of Helicobacter pylori CagA that underpin gastric carcinogenesis. *Cell Mol Immunol* 2020; 17: 50-63.
38. Osaki T, Okuda M, Ueda J, *et al.* Multilocus sequence typing of DNA from faecal specimens for the analysis of intra-familial transmission of Helicobacter pylori. *J Med Microbiol* 2013; 62: 761-765.
39. Salih BA. Helicobacter pylori infection in developing countries: the burden for how long? *Saudi J Gastroenterol* 2009; 15: 201-207.
40. Leja M, Axon A, Brenner H. Epidemiology of Helicobacter pylori infection. *Helicobacter* 2016; 21 (Suppl 1): 3-7.
41. Yahaghi E, Khamesipour F, Mashayekhi F, *et al.* Helicobacter pylori in vegetables and salads: genotyping and antimicrobial resistance properties. *Biomed Res Int* 2014; 2014: 757941. doi:10.1155/2014/757941
42. Tirodimos I, Bobos M, Kazakos E, *et al.* Molecular detection of Helicobacter pylori in a large Mediterranean river, by direct viable count fluorescent in situ hybridization (DVC-FISH). *J Water Health* 2014; 12: 868-873.
43. Queralt N, Bartolome R, Araujo R. Detection of Helicobacter pylori DNA in human faeces and water with different levels of faecal pollution in the north-east of Spain. *J Appl Microbiol* 2005; 98: 889-895.
44. Mazari-Hiriart M, Lopez-Vidal Y, Castillo-Rojas G, Ponce de Leon S, Cravioto A. Helicobacter pylori and other enteric bacteria in freshwater environments in Mexico City. *Arch Med Res* 2001; 32: 458-467.
45. Khoshdel A, Kasiri KA , Dehkordi SV, Lotfizadeh M, Hosseinzadeh S, Rabie AA. Finding the existence of of Helicobacter pylori in springs of Chaharmahal and Bakhtiari Province, Iran by polymerase chain reaction. *Int J Epidemiol Res* 2016; 3: 345-350.
46. Figura N, Vindigni C, Covacci A, *et al.* CagA positive and negative Helicobacter pylori strains are simultaneously present in the stomach of most patients with non-ulcer dyspepsia: relevance to histological damage. *Gut* 1998; 42: 772-778.
47. Slater E, Owen RJ., Williams M, Pounder RE. Conservation of the cag pathogenicity island of Helicobacter pylori:

associations with vacuolating cytotoxin allele and IS605 diversity. *Gastroenterology* 1999; 117: 1308-1315.
48. Chen XJ, Yan J, Shen YF. Dominant cagA/vacA genotypes and coinfection frequency of H. pylori in peptic ulcer or chronic gastritis patients in Zhejiang Province and correlations among different genotypes, coinfection and severity of the diseases. *Chin Med J* 2005; 118: 460-467.
49. Amirhooshang A, Ramin A, Ehsan A, Mansour R, Shahram B. High frequency of Helicobacter pylori DNA in drinking water in Kermanshah, Iran, during June-November 2012. *J Water Health* 2014; 12: 504-512.
50. Basic Local Alignement Search Tool. U.S. National Library of Medicine https://blast.ncbi.nlm.nih.gov/Blast.cgi].
51. Regulation of the Minister of Health of 13 November 2015 on the Quality of Water Intended for Human Consumption. Journal of Laws 2015 No. 1989. In Polish. [https://dziennikustaw.gov.pl/DU/rok/2015/pozycja/1989].
52. [List of Polish Standards] [https://www.pkn.pl/polskie-normy/wykazy-pn].
53. Khan A, Farooqui A, Kazmi SU. Presence of Helicobacter pylori in drinking water of Karachi, Pakistan. *J Infect Dev Ctries* 2012; 6: 251-255.
54. Watson CL, Owen RJ, Said B, *et al.* Detection of Helicobacter pylori by PCR but not culture in water and biofilm samples from drinking water distribution systems in England. *J Appl Microbiol* 2004; 97: 690-698.
55. Brooks HJ, Ahmed D, McConnell MA, Barbezat GO. Diagnosis of Helicobacter pylori infection by polymerase chain reaction: is it worth it? *Diagn Microbiol Infect Dis* 2004; 50: 1-5. doi: 10.1016/j.diagmicrobio.2003.11.010
56. Samra ZQ, Javaid U, Ghafoor S, Batool A, Dar N, Athar MA. PCR assay targeting virulence genes of Helicobacter pylori isolated from drinking water and clinical samples in Lahore metropolitan, Pakistan. *J Water Health* 2011; 9: 208-216.
57. West AP, Millar MR, Tompkins DS. Effect of physical environment on survival of Helicobacter pylori. *J Clin Pathol* 1992; 45: 228-223.
58. Santiago P, Moreno Y, Ferrus MA. Identification of viable Helicobacter pylori in drinking water supplies by cultural and molecular techniques. *Helicobacter* 2015; 20: 252-259.
59. Hortelano I, Moreno Y, Vesga FJ, Ferrus MA. Evaluation of different culture media for detection and quantification of H. pylori in environmental and clinical samples. *Int Microbiol* 2020; 23: 481-487.
60. Krzysiek-Maczka G, Targosz A, Ptak-Belowska A, *et al.* Molecular alterations in fibroblasts exposed to Helicobacter pylori: a missing link in bacterial inflammation progressing into gastric carcinogenesis? *J Physiol Pharmacol* 2013; 64: 77-87.
61. Krzysiek-Maczka G, Targosz A, Szczyrk U, *et al.* Role of Helicobacter pylori infection in cancer-associated fibroblast-induced epithelial-mesenchymal transition in vitro. *Helicobacter* 2018; 23: e12538. doi: 10.1111/hel.12538
62. Krzysiek-Maczka G, Wrobel T, Targosz A, *et al.* Helicobacter pylori-activated gastric fibroblasts induce epithelial-mesenchymal transition of gastric epithelial cells in vitro in a TGF-β-dependent manner. *Helicobacter* 2019; 24: e12653. doi: 10.1111/hel.12653
63. Krzysiek-Maczka G, Targosz A, Szczyrk U, Strzalka M, Brzozowski T, Ptak-Belowska A. Involvement of epithelial-mesenchymal transition-inducing transcription factors in the mechanism of Helicobacter pylori-induced fibroblasts activation. *J Physiol Pharmacol* 2019; 70: 727-736.
64. Krzysiek-Maczka G, Targosz A, Szczyrk U, *et al.* Long-term Helicobacter pylori infection switches gastric epithelium reprogramming towards cancer stem cell-related differentiation program in Hp-activated gastric fibroblast-TGF-β dependent manner. *Microorganisms* 2020; 2: 1519. doi: 10.3390/microorganisms8101519
65. Iwanczak B, Laszewicz W, Iwanczak F, *et al.* Genotypic and clinical differences of seropositive Helicobacter pylori children and adults in the Polish population. *J Physiol Pharmacol* 2014; 65: 801-807.

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