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Potential role of beavers (*Castor fiber*) in contamination of water in the Masurian Lake District (north-eastern Poland) with protozoan parasites *Cryptosporidium* spp. and *Giardia duodenalis*

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Abstract

The purpose of this study was to assess the possible influence of beavers on the contamination of lake water with zoonotic parasites *Giardia duodenalis* and *Cryptosporidium* spp., with respect to the risk to human health. A total of 79 water samples were taken around the habitats of beavers from 14 localities situated in the recreational Masurian Lake District (north-eastern Poland). Water was sampled in the spring and autumn seasons, at different distances from beavers' lodges (0-2, 10, 30, and 50 m). The samples were examined for the presence of (oo)cysts of zoonotic protozoa *Giardia duodenalis* and *Cryptosporidium* spp. by direct fluorescence assay (DFA) and by nested and real time PCR. By DFA, the presence of *Giardia* cysts was found in 36 samples (45.6%) and the presence of *Cryptosporidium* oocysts in 26 samples (32.9%). Numbers of *Giardia* cysts, *Cryptosporidium* oocysts, and summarised (oo)cysts of both parasites showed a significant variation depending on locality. The numbers of *Giardia* cysts significantly decreased with the distance from beavers' lodges while the numbers of *Cryptosporidium* oocysts did not show such dependence. The amount of *Giardia* cysts in samples collected in spring was approximately 3 times higher than in autumn. Conversely, a larger number of *Cryptosporidium* oocysts were detected in samples collected in autumn than in spring. By PCR, *Giardia* DNA was found in 38 samples (48.1%) whereas DNA of *Cryptosporidium* was found in only 7 samples (8.9%). Eleven *Giardia* isolates were subjected to phylogenetic analysis by restriction fragment length polymorphism PCR or sequencing which evidenced their belonging to zoonotic assemblages: A (3 isolates) and B (8 isolates). In conclusion, water in the vicinity of beavers' lodges in the tested region was markedly contaminated with (oo)cysts of *Giardia duodenalis* and *Cryptosporidium* spp., which confirms the potential role of beavers as a reservoir of these parasites and indicates a need for implementation of appropriate preventive measures to protect tourists' health.

Keywords: beavers, *Giardia*, *Cryptosporidium*, Masurian lakes, Poland.

Introduction

The parasitic protozoa *Cryptosporidium* spp. and *Giardia duodenalis* may pose a serious threat to human and animal health (5, 10). These parasites cause symptoms of gastroenteritis (diarrhoea, abdominal pain, vomiting, weakness, and dehydration). In Poland, cryptosporidiosis cases among immunocompetent persons are rarely noticed (4 cases in 2012-13) (15). However, a higher percentage of infections was found among people with immune deficiency (36%) (2, 15). In Poland, cases of giardiasis are noted much more often (approximately 1800 per year) (16), which may be due to highly-developed laboratory diagnostics in this field.

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Water intended for human consumption or recreation may be an important source of infection when contaminated with oocysts or cysts of these parasites (5, 10). The presence of parasites in water represents a particular hazard in the areas attractive to tourists, such as the Masurian Lake District.

One of the important sources of parasitic protozoa in surface waters are semi-aquatic mammals, especially beavers (*Castor fiber*) (7-9). Currently, the estimated beaver population in Poland is about 50 000 individuals, localised mainly in the north-eastern part of the country (6). Only a small number of (oo)cysts are sufficient to cause human outbreaks. Furthermore, dispersion forms of *Giardia* and *Cryptosporidium* are resistant to chlorination of water, and standard methods of water treatment do not reduce their threat. Thus, the proximity of the habitats of beavers and other semi-aquatic mammals to recreational areas in the Masurian Lake District poses a potential risk to human health.

The purpose of this study was to assess the possible influence of beavers on the contamination of lake water with zoonotic parasites *Giardia duodenalis* and *Cryptosporidium* spp., regarding the threat to human health.

Material and Methods

Sampling. The study was conducted in 2010-2014, in cooperation with the Research Station of Ecological Agriculture and Preserve Animal Breeding of the Polish Academy of Sciences in Popielno (Masurian region, Poland), which organised water sampling. Water samples (each of 50 L) were collected from 14 beaver habitats localised on 12 selected Masurian lakes and one forest watercourse (Table 1, Fig. 1). For each of the beaver habitats 3 water sampling zones were marked: I – a close distance from the animals' lodge (0–2 m), II – 10 m from the beavers' lodge, and III – about 30–50 m (depending on the size of the lake) from the lodge (Fig. 2). In total, 150 L of water were collected from all zones. Samples from each beaver habitat were collected twice a year (excluding the forest watercourse, where samples were taken only once).

Table 1. Data of sampling localities

No	Lake	GPS localisation	Area
1	Śniardwy (A)	N53˚45.875', E021˚37.871'	11340.4 ha
2	Śniardwy (B)	N53˚45.866', E021˚37.891'	
3	Nidzkie	N53˚33.160', E021˚38.494'	1818 ha
4	Wesołek	N53˚38.520', E021˚38.994'	6 ha
5	Chudek (Wyłuszczarnia)	N53°38'360', E021°34.298'	1.1 ha
6	Gaik (Guzianka)	N53˚38.601', E021˚34.512'	1.2 ha
7	Oczko	N53°35.123', E021°33.153'	10.5 ha
8	Mojcz Wielki	N53˚46.228', E021˚27.656'	164 ha
9	Mokre	N53˚41.934', E021˚22.830'	846 ha
10	Płociczno	N53°47.580', E021°29.541'	21.5 ha
11	Dłużec (A)	N53°42.556', E021°32.215'	126.9 ha
12	Dłużec (B)	N53°47.541', E021°29.590'	
13	Flosek	N53°44.567'; E021°33.273'	3 ha
14	forest watercourse (F.w.)	N53°41.232'; E021°36.550'	N/A

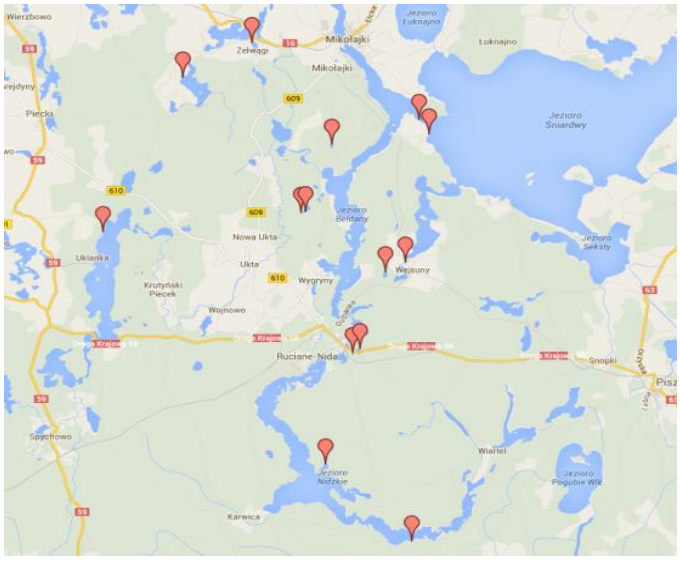


Fig. 1. The area under study

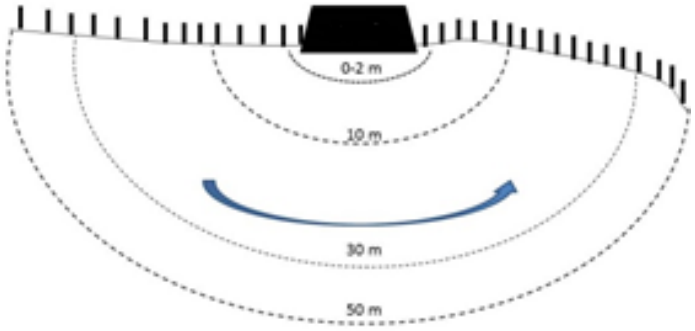


Fig. 2. The zones of water sampling near a beaver's lodge

Microscopic examination. Water samples were filtered, and next immunomagnetic separation (IMS) and direct fluorescence assay (DFA) were performed based on the protocol of the American Environmental Protection Agency, US EPA Method 1623 (25).

Filtration. Water samples (each of 50 L volume) were filtered under laboratory conditions using an Envirochek capsule filter (Pall, USA) and peristaltic pump with flow control. After filtration, filters were filled with wash buffer, shaken, the filter contents were centrifuged, and the obtained pellet was washed twice. The procedure was performed according to the filter manufacturer's instructions.

Immunomagnetic separation. The pellet obtained after filtration was processed through immunomagnetic separation **(**IMS). The aim of the IMS was to determine the concentration of *Giardia duodenalis* and *Cryptosporidium* spp. (oo)cysts with the use of monoclonal antibodies. The IMS was performed using the GC-COMBO Dynabeads commercial kit (Life Technologies, part of Thermo Fisher Scientific, USA), mixer, Leighton's test tubes, and magnetic concentrators.

Direct fluorescence assay. Direct fluorescence assay (DFA) was performed using an Aqua-Glo™ commercial test (Waterborne Inc., USA). IMS product in 15 µL volume was added to each microscopic well and allowed to dry at room temperature. The samples were next fixed in 50 µL of methanol and allowed to dry. After that, 50 µL of 4'6-diamidino-2-phenyl indole (DAPI) in PBS (0.4 µg DAPI/mL) was dropped in and left for 4 min at room temperature. Following removal of the DAPI, the slides were rinsed in 100 µL of wash buffer and left for 1 min. Then the wash buffer was removed, and 50 µL of conjugate anti-*Cryptosporidium*/*Giardia* with fluorescein isothio-cyanate (FITC) was placed on each well. The slides were placed in a humidified chamber and incubated at 37°C for 30 min. Then a washing step was performed as described above. One drop of BlockOut™ counterstain (Waterborne Inc., USA) was added to each well to reduce non-specific background fluorescence and slides were incubated for 1 min at room temperature. After washing (as above) and drying the slides, a drop of No-Fade™ mounting medium (Waterborne Inc., USA) was placed in each well, covered by glass, and viewed under epifluorescence microscope (×400) (Olympus BX51, Olympus Optical Co. Ltd, Tokyo). *Giardia* cysts and *Cryptosporidium* oocysts were identified on the basis of their size, shape, and structure according to guideline described in the EPA 1623 method (25). Positive and negative controls were used. The results were reported as densities of the *Giardia* cysts and *Cryptosporidium* oocysts per 100 L of water or as prevalence, *e.g*. the percentage of positive habitats among the total examined.

DNA extraction and PCR. Part of the IMS product (35 µL) was subjected to DNA extraction. The samples underwent 7 freeze-thawing cycles of liquid nitrogen immersion followed by heating at 65°C. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions, pursuant to which samples were lysed with proteinase K overnight. The extracted DNA was stored at -20ºC until PCR assay.

For *Giardia* detection, semi-nested PCR of the fragment of *β-giardin* gene (384 bp) was performed according to a procedure by Cacciò *et al.* (4) with minor modifications. Each reaction mixture (50 µL) contained: 10 pmol of each primer; 0.2 mM of each dNTP (Fermentas, Lithuania); 50 mM KCl, 10 mM Tris-HCl (pH 9.0); 2 mM $MgCl_2$; 1.5 U of *Taq* DNA polymerase (Qiagen, Germany), and 1-2 µL of DNA. Negative and positive controls were included in each PCR. Amplification was performed as follows: an initial cycle at 94ºC for 5 min; followed by 35 cycles at 94ºC for 30 s, at 65ºC for 30 s, and at 72ºC for 60 s. The final extension was done at 72ºC for 7 min.

For detection of *Cryptosporidium* spp., the nested PCR method described by Xiao *et al.* (26) with modifications by Santin *et al.* (19) was used. The mixture (50 µL) in the first reaction contained: 3 mM $MgCl_2$; 0.2 mM dNTP (Fermentas, Lithuania), 2.5 U of Taq DNA polymerase (Qiagen, Germany), and 1 µM of each primer. Negative and positive controls were included in each PCR. Amplification was performed under the following conditions: an initial cycle at 94ºC for 3 min; followed by 35 cycles at 94ºC for 45 s, at 59ºC for 45 s, and at 72ºC for 60 s; and a final extension at 72ºC for 7 min. In the second reaction, the

composition of the PCR mixture was the same, except for the concentration of $MgCl_2$ (1.5 mM). The parameters of the reaction were as follows: initially at 94ºC for 3 min; followed by 40 cycles at 94ºC for 30 s, at 58ºC for 90 s, and at 72ºC for 120 s; and a final extension at 72ºC for 7 min. Amplification was performed using a TProfessional 48 thermocycler (Biometra, Germany). Each semi-nested PCR product was subjected to electrophoresis (in 1.5% agarose gel stained with ethidium bromide). PCR products were purified by spin column (QIAquick Purification Kit, Qiagen, Germany).

Real time PCR. Real time PCR examination was performed using the method according to Guy *et al.* (11). For the detection of *Giardia* DNA, the fragments of *β-giardin* gene were amplified using the following primers: Giardin P241, Giardin P434 (P1), Giardin P434 (H3), and TaqMan probes. For the detection of *Cryptosporidium* DNA, the fragment gene of COWP was amplified, using P702 primers and TaqMan probe. Each reaction mixture (25 µL) contained: 12.5 µL of mastermix (IQ Supermix, Bio-Rad, USA); 2.5 µL of each primer (5 µM); 2.5 µL of TaqMan probe (2 µM), and 5 µL of DNA. Negative and positive controls were included in each PCR. The test was performed in a CFX-96 thermocycler (Bio-Rad, USA). Reaction parameters: initial activation of the polymerase at 95°C for 10 min; and 40 two-step cycles at 95°C for 15 s and at 60°C for 1 min. The threshold value (CT) for positive fluorescence, representing a multiple of the basis level of fluorescence signal in the first cycles of PCR, was determined automatically by the software.

Analysis of sequences and phylogenetics. Selected amplicons of the nested PCR were sequenced on both strands using the BIGDYE Cycle Sequencing Kit (Life Technologies, USA) according to the manufacturer's instructions. Chromatograms were examined using BioEdit (http://www.mbio.ncsu.edu/BioEdit/), and nucleotide sequences were searched for similarities using BLAST (http://www.ncbi.nlm.nih.gov/blast/). Multiple alignments were generated using ClustalW (23) and included sequences from the present work and reference sequences retrieved from GenBank. A nucleotide distance pairwise matrix calculation was performed using MEGA5.1 (22) software. PhyML version 3.1 was used to perform maximum likelihood analyses with the JC69 substitution model. *Giardia muris* was used as an outgroup. The confidence of grouping was assessed by bootstrapping, using 1000 replicates. Formation of one polytomic tree was conducted in MEGA5.1 (22) by creating a consensus tree at the 20% cut-off level. Selected nucleotide sequences have been deposited in GenBank under the following accession numbers: KJ475125, KJ475126, KJ475127, KJ475128, and KJ475129.

Statistical analysis. Statistical analysis was performed by χ^2 test and Mann-Whitney *U* test with the use of STATISTICA v. 6.0 package (Statsoft, USA).

Results

DFA results. Overall, among 79 water samples examined in DFA, in 36 (45.6%) low amounts of *Cryptosporidium* oocysts were observed, while in 26 samples (32.9%) numerous cysts of *Giardia* were found. The presence of both parasites was found in 12 samples (15.2%). In total, in water samples collected from 14 beaver habitats located on 12 surface waters, 149 dispersion forms of *Giardia* and *Cryptosporidium* were found (including 71 *Giardia* cysts and 78 *Cryptosporidium* oocysts). Most parasites of *Giardia* and *Cryptosporidium* were found in samples taken from Lakes Płociczno and Dłużec (A) whereas the lowest amounts of parasites were found in samples from Lakes Mokre and Dłużec (B). *Giardia* occurred most frequently in samples from Lakes Płociczno and Dłużec (A). *Cryptosporidium* occurred most frequently in the samples from Lakes Nidzkie, Mojcz Wielki, and Chudek (Table 2). Densities of *Giardia* cysts, *Cryptosporidium* oocysts, and all (oo)cysts of both parasites showed significant variation depending on locality (χ^2>45.0, P<0.0001). The numbers of *Giardia* detected in one slide ranged from 1 to 21 cysts (mean 2.9 cysts) and the numbers of *Cryptosporidium* ranged from 1 to 9 per slide (mean 2.2 oocysts).

Overall in DFA, the highest density of both *Giardia* and *Cryptosporidium* (oo)cysts was found in samples collected in the 10 m zone, slightly less was found in the 0–2 m zone and the lowest number in the 30–50 m zone (Table 3). The densities of all (oo)cysts of both parasites did not differ significantly by zone (χ^2 = 1.502, P = 0.472). The prevalence of habitats in which both parasites were found was the highest for the 0–2 m zone, less for the 30–50 m zone, and the lowest for the 10 m zone (Table 4). *Giardia* occurred most frequently in the 10 m zone, less in the 0–2 m zone, and least frequently in the 30–50 m zone (Table 3). The densities of *Giardia* cysts showed a significant variation by zone (χ^2 = 7.918, P = 0.019). The prevalence of habitats where *Giardia* was found was the highest for the 0–2 m zone and the lowest for the 30–50 m zone (Table 4). *Cryptosporidium* was found most frequently in the 30–50 m zone, while less frequently and in equal numbers, in the 10 m and 0–2 m zones (Table 3). The densities of *Cryptosporidium* oocysts did not differ significantly by zone (χ^2 = 2.512, P = 0.285). The prevalence of habitats in which *Cryptosporidium* was found was the highest for the 0–2 m and 30–50 m zones, and the lowest for the 10 m zone (Table 4).

Seasonal dependence. The density of *Giardia* cysts in samples collected in spring was nearly 4 times higher than in autumn (mean values were respectively 59.2 and 17.1 cysts per 100 L of water), while a larger number of *Cryptosporidium* oocysts were detected in samples collected in autumn (mean 55.8 oocysts/100 L) than in spring (mean 30.6 oocysts/100 L) (Table 5, Figs 3 and 4). These seasonal differences were,

however, significant neither for *Giardia* (U = 75.0, P = 0.340) nor for *Cryptosporidium* (U = 56.5, P = 0.088).

PCR results. *Giardia* DNA was found in 38 of 79 samples (48.1%) by nested and/or real-time PCR (RT-PCR). In total, 27 samples were positive in nested PCR and 24 samples in RT-PCR. DNA of *Cryptosporidium* was found in seven samples (8.9%), only in RT-PCR.

Giardia DNA was detected in largest numbers in samples from Lakes Mokre (6 samples), Płociczno (4 samples), and Chudek (4 samples), and least frequently in samples from Lake Śniardwy (1 sample). The largest numbers of positive results for *Giardia* in both tests were found in samples taken from Lakes Płociczno (4 samples), Dłużec (A) (3 samples), and Dłużec (B) (3 samples). *Cryptosporidium* DNA was found in 3 samples from Lake Wesołek and in one sample each from the Lakes Mokre, Dłużec (A), Gaik, and Flosek. Restricted fragment length polymorphism PCR (RFLP-PCR) analysis showed that 6 samples (strong positive samples in electrophoresis) belonged to assemblage A (3 samples) and assemblage B (3 samples) (Table 6). The sequencing of the next 5 samples showed the assemblage B of *Giardia*. Due to negative or weak positive results in nested PCR, or due to positive results only in RT-PCR, the rest of the PCR-positive samples were not analysed by RFLP or sequencing.

Phylogenetic analysis. Among 27 samples positive in nested PCR, 5 sequences were selected for phylogenetic analysis. These sequences were deposited in the GenBank database under the following accession numbers: KJ475125, KJ475126, KJ475127, KJ475128, and KJ475129. The phylogenetic analysis of the sequence of a fragment of *β-giardin* gene from water samples and the reference sequences for each type of genetic *Giardia duodenalis* showed the presence of assemblage B of *Giardia* in the examined samples. The sequence of the isolate from Lake Chudek was identical to the reference sequence for sub-assemblage B3. The sequences derived from Lakes Flosek and Dłużec (B) and the forest watercourse were the same and most closely related to sub-assemblages B1 and B3. The sequence from Dłużec (A) reveals single nucleotide polymorphism (SNP), which was not observed in the other isolates. It should be noted that the obtained isolates formed a clearly separated clade. In Lake Dłużec–two different isolates were found, which may indicate the presence of two different variants of the examined gene in the studied population of *Giardia* (Fig. 5).

Table 2. Densities of *Giardia* and *Cryptosporidium* (oo) cysts in water of individual localities estimated by DFA

Locality	Density of *Giardia* cysts per 100 L	Density of *Cryptosporidium* cysts per 100 L	Total density of (oo)cysts per 100 L
Śniardwy (A)	37.0	22.2	59.2
Śniardwy (B)	44.4	14.8	59.2
Nidzkie	0	96.2	96.2
Wesołek	37.0	44.4	81.4
Chudek	7.4	88.8	96.2
Gaik	7.4	29.6	37.0
Oko	44.4	59.2	103.6
Mojcz Wielki	7.4	96.2	103.6
Mokre	7.4	7.4	14.8
Płociczno	214.6	22.2	236.8
Dłużec (A)	96.2	51.8	148.0
Dłużec (B)	7.4	14.8	22.2
Flosek	7.4	29.6	37.0
forest watercourse	7.4	0	7.4
Mean ± S.E.	37.5 ± 15.3	41.2 ± 8.8	78.7 ± 16.3

Table 3. Densities of the parasites per 100 L of water detected by DFA in selected sampling zones (mean values ± SD for spring and autumn)

Zone	*Giardia* cysts	*Cryptosporidium* oocysts	Total (oo)cysts
0–2 m	15.3 ± 25.4	11.6 ± 8.1	13.5 ± 18.6
10 m	18.8 ± 46.3	12.5 ± 19.7	15.7 ± 35.0
30–50 m	5.0 ± 8.2	19.4 ± 23.7	12.2 ± 18.8

Table 4. Prevalence of the positive beaver habitats, expressed as positive/total (percentage) where parasites were detected by DFA in individual zones (total values for spring and autumn)

Zone	*Giardia* cysts	*Cryptosporidium* oocysts
0–2 m	12/14 (85.7%)	14/14 (100%)
10 m	8/14 (57.1%)	8/14 (57.1%)
30–50 m	6/14 (42.9%)	14/14 (100%)

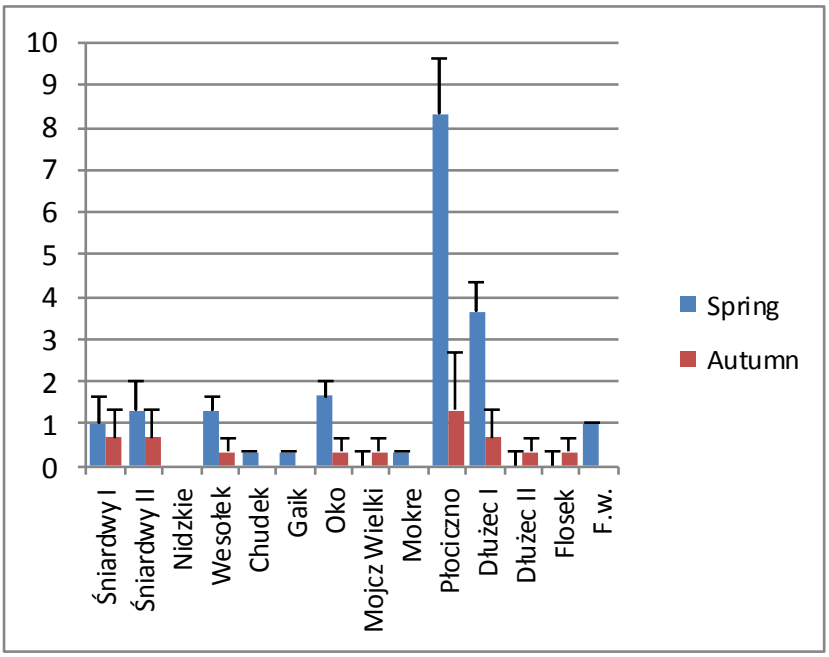


Fig. 3. Seasonal dependence of *Giardia* cysts recorded by DFA (mean SD)

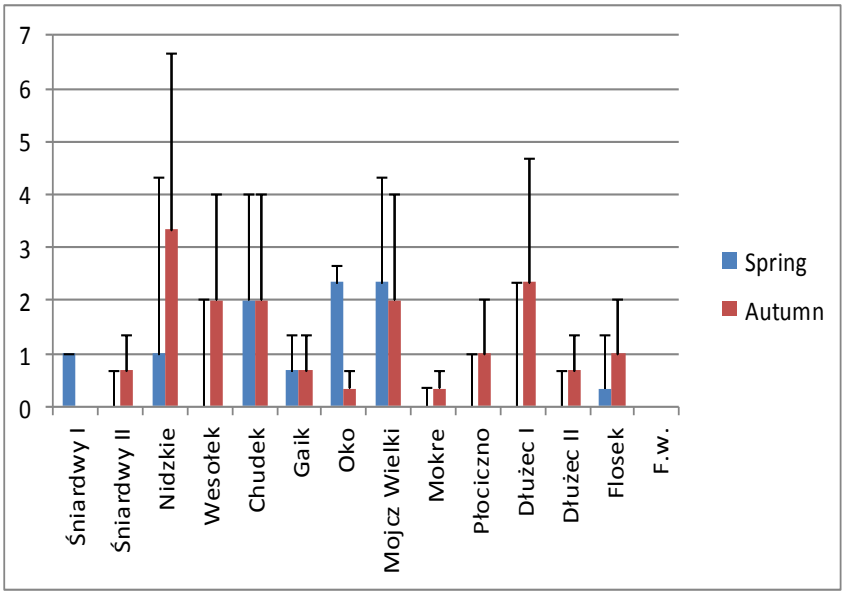


Fig. 4. Seasonal dependence of *Cryptosporidium* oocysts recorded by DFA (mean SD)

Table 5. Seasonal variability in the density of *Giardia* and *Cryptosporidium* parasites in water of examined lakes

Lake	Density of *Giardia duodenalis* cysts per 100 L		Density of *Cryptosporidium*. oocysts per 100 L	
	Spring	Autumn	Spring	Autumn
Śniardwy (A)	44.4	29.6	44.4	0
Śniardwy (B)	59.2	29.6	0	29.6
Nidzkie	0	0	44.4	148.0
Wesołek	59.2	14.8	0	88.8
Chudek	14.8	0	88.8	88.8
Gaik	14.8	0	29.6	29.6
Oko	74.0	14.8	103.6	14.8
Mojcz Wielki	0	14.8	103.6	88.8
Mokre	14.8	0	0	14.8
Płociczno	370.0	59.2	0	44.4
Dłużec (1)	162.8	29.6	0	103.6
Dłużec (2)	0	14.8	0	29.6
Flosek	0	14.8	14.8	44.4
forest watercourse	14.8	N.A.	0	N.A
Mean ± S.E.	59.2 ± 26.7	17.1 ± 4.7	30.6 ± 10.8	55.8 ± 12.1

Table 6. The results of detection of *Giardia duodenalis* and *Cryptosporidium* in water samples by PCR

Locality	*Giardia duodenalis*							*Cryptosporidium* spp.						
	0-2 m		10 m		30-50 m		Σ results +	0-2 m		10 m		30-50 m		Σ results +
	Spring	Autumn	Spring	Autumn	Spring	Autumn		Spring	Autumn	Spring	Autumn	Spring	Autumn	
Śniardwy (A)	+ (A type)#	(-)	(-)	(-)	(-)	(-)	1	(-)	(-)-	(-)	(-)	(-)	(-)	0
Śniardwy (B)	(-)	(-)	+	(-)	(-)	(-)	1	(-)	(-)	(-)	(-)	(-)	(-)	0
Nidzkie	(-)	+ (A type)#	(-)	(-)	(-)	+ (A type)#	2	(-)	(-)	(-)	(-)	(-)	(-)	0
Wesołek	(-)	(-)	(-)	(-)	(-)	(-)	0	(-)	(-)	(-)	+	+	+	3
Chudek	(-)	(-)	+ (B type)*	+	+ (B type)#	+	4	(-)	(-)	(-)	(-)	(-)	(-)	0
Gaik	+ (B type)#	(-)	(-)	(-)	+ (B type)#	(-)	2	(-)	(-)	+	(-)	(-)	(-)	1
Oko	+	(-)	+	(-)	+	(-)	3	(-)	(-)	(-)	(-)	(-)	(-)	0
Mojcz Wielki	+	+	+	(-)	(-)	(-)	3	(-)	(-)	(-)	(-)	(-)	(-)	0
Mokre	+	+	+	+	+	+	6	(-)	(-)	(-)	+	(-)	(-)	1
Płociczno	+	(-)	+	(-)	+	+	4	(-)	(-)	(-)	(-)	(-)	(-)	0
Dłużec (A)	+ (B type)*	(-)	+	(-)	+	(-)	3	+	(-)	(-)	(-)	(-)	(-)	1
Dłużec (B)	+	(-)	+ (B type)*	(-)	+	(-)	3	(-)	(-)	(-)	(-)	(-)	(-)	0
Flosek	+ (B type)*	(-)	+	+	+	+	5	(-)	(-)	(-)	+	(-)	(-)	1
forest watercourse	+ (B type)*	N.A.	N.A.	N.A.	N.A.	N.A.	1	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0
Total positive	10	3	9	3	8	5	38	1	0	1	3	1	1	7

*results of sequencing; # RFLP-PCR analysis; N.A. – not applicable

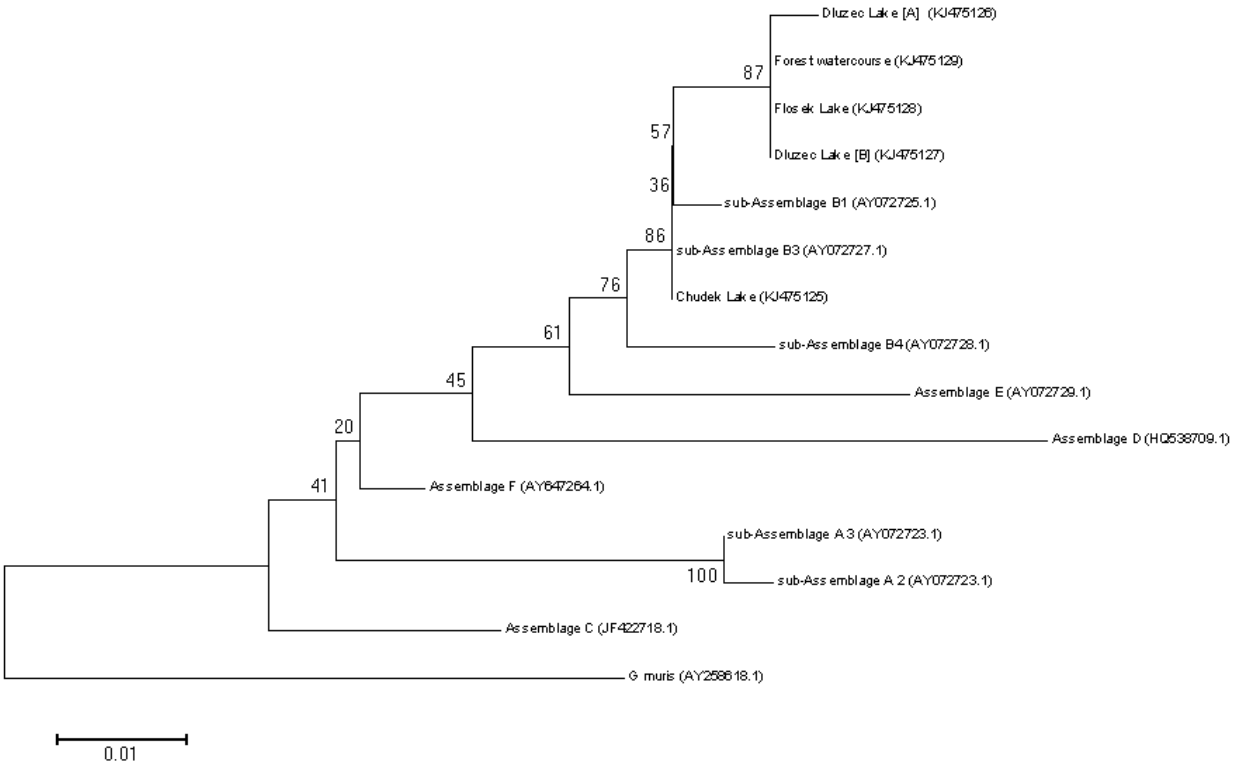


Fig. 5. Phylogenetic analysis of sequences of *β-giardin* gene in water samples

Discussion

Farmed and free-living animals excrete the invasive forms of *Cryptosporidium* and *Giardia* into the environment with faeces. Semi-aquatic animals are recognised as important sources of these parasites (5, 10). Because the population of beavers in Poland is large, this species may have the biggest epidemiologic importance, especially in areas abounding in surface waters. So far, the studies conducted in Poland have shown *Cryptosporidium* infection in 32% of beavers and 58% of muskrats (*Ondatra zibethicus*). Infection with *Giardia* has been noted in 4.5% of beavers and 87% of muskrats (2, 18). In other countries, a considerable degree of *Cryptosporidium* and *Giardia* infection in this group of animals has also been reported (7, 8, 9). In Poland, only a few studies have focused on the occurrence of *Cryptosporidium* and *Giardia* in water; the presence of these protozoa in surface waters has been demonstrated in the regions of Poznań, Gdańsk, Sopot, and Gdynia (2).

The information about the contamination of the water of the Masurian Lakes by parasitic protozoa is scarce. Recently a study performed by Bajer *et al.* (3), who examined 94 water samples from Polish rivers, lakes, and water treatment plants with the use of IFA and PCR, demonstrated *Cryptosporidium* oocysts in 85% of surface water and in 59% of raw (intake) water samples. *Giardia* cysts were observed in 61% of surface water and in 6% of raw water samples (3). Despite data supporting the importance of semi-aquatic animals in the epidemiology of *Cryptosporidium* and *Giardia* infections (13), there is also no information about how much these animals contribute directly to the contamination of water with parasitic protozoans and what the range of contamination is around the animal habitats, with respect to the health risk for people entering the water.

In the present study, the presence of parasitic protozoa (*Giardia* and *Cryptosporidium*) in all tested areas of water around the beavers' lodges was found. The largest densities of *Giardia* and *Cryptosporidium* were found in Lakes Płociczno and Dłużec. In samples from other lakes fewer (oo)cysts in the DFA were observed.

A significant relationship between the distance from the beavers' lodge and the density of (oo)cysts in the water was observed in the case of *Giardia*, but not in the case of *Cryptosporidium*. The largest densities of *Giardia* cysts were found in samples taken in proximity to beavers' lodges where the depth of water was low (the 0–2 m zone), and the smallest densities of them in the area of 30–50 m. This may indicate that beavers were possibly the source of these parasites in the water. In the case of *Cryptosporidium*, the largest number of oocysts was observed in the 30–50 m zone, which may suggest that the beavers may not be directly responsible for the contamination of water in the studied areas. There is also a possibility that due to much smaller size and mass, the dispersion forms migrate further into the lake, compared to *Giardia* cysts.

Seasonal differences between the numbers of *Giardia* and *Cryptosporidium* parasites in water were observed, which, however, did not attain the significance level. More frequent detection of parasites of the genus *Giardia* in spring than in autumn could be affected by such factors as increased turbidity of water in spring, which favours the persistence of parasites dispersed in water (above the bottom) and reduces the impact of water movements on migration; and increased frequency and intensity of *Giardia* infection in beavers in the spring because of the presence of young individuals susceptible to infection. In contrast, the greater detection of *Cryptosporidium* than *Giardia* in samples taken in autumn could be affected by the longer survival period of *Cryptosporidium* oocysts (about 8 months) compared to *Giardia* cysts (about 3 months), which could result in longer persistence in water and longer microscopic detectability.

However, the obtained results did not show full agreement between DFA and PCR. These differences could be attributed to the small number of parasites in the samples and/or to the only partial clearance of PCR inhibitors. In particular, a very low number of *Cryptosporidium* oocysts in samples and the possibility of the presence of dead oocysts with destroyed genetic material could be reasons for negative results in PCR. The differences between DFA and PCR may result also from low DFA specificity, which led to an overestimation of the number of (oo)cysts in water by cross-reaction with non-target organisms.

In our study, molecular diagnostics allowed the detection of sequences of partial β-giardin gene of *G. intestinalis* in water samples from 5 lakes. To conduct phylogenetic analysis, the reference sequences previously obtained by various research groups (4, 12, 14, 17) and deposited in GenBank, were selected. Reference sequences of *G. intestinalis* were isolated from stool samples derived from humans – genotypes A2, A3, B1, B2, and B4; cattle *(Bos taurus)* – genotype E; dog *(Canis familiaris)* – genotype C; raccoon dog *(Nyctereutes procyonoides)* –genotype D, and cat *(Felis catus)* – genotype F. The reference sequences of *G. muris* isolated from mouse *(Mus musculus)* were also used. The analysis of RFLP-PCR and/or sequencing the isolates from Lakes Śniardwy (A) and Nidzkie showed *Giardia* assemblage A (zoonotic), while from Lakes Chudek, Gaik, Dłużec (A), and the forest watercourse, assemblage B of *Giardia* (also zoonotic). The occurrence of zoonotic assemblages of *Giardia* in beavers has also been confirmed by other authors (1, 21, 24). Comparing sequences obtained from Masurian lakes with sequences obtained from the North American beaver *(Castor canadensis)* (GenBank: DQ789116) showed similar genotypes. However, detailed analysis showed 2-3 SNPs differentiating these isolates. Genetic variability might suggest the occurrence of separate *G. intestinalis* populations, but extending the analysis to other sequences from environmental samples (GenBank: KC748021, DQ116609, and FN377868) derived from various countries worldwide makes this hypothesis questionable, because genetic variability on the internal and external assemblage levels has been also detected. In this context, the source of parasitic contamination of environmental samples may often remain undetermined.

In the present study, the estimated densities of *Giardia* cysts per 100 L of water ranged from 0.0 to 214.6, and of *Cryptosporidium* oocysts from 0.0 to 96.2. These figures were higher compared to corresponding values found in the samples of surface waters at recreational sites where no beavers exist, such as Amsterdam (20), where they ranged from 0.0 to 12.0 and from 0.0 to 6.0 per 100 L of water respectively. The maximum concentrations of these parasites in water found in our survey represent infectious doses for humans (21). Therefore, the obtained results specifying the degree of water contamination by parasitic protozoa (*Cryptosporidium* and *Giardia*) in the proximity of beaver habitats in the area contiguous with tourist areas may suggest that these animals could probably be one of the potential factors that contribute to the pollution of lake water with parasitic protozoans. Therefore, the presented results might also have, besides their scientific importance, a practical significance for the implementation of appropriate preventive measures to protect human health in the recreation areas of the Masurian Lakes.

Conflict of Interests Statements: The authors declare that there is no conflict of interest regarding the publication of this article.

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