Vol. 84: 191–203, 2020
https://doi.org/10.3354/ame01936

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online June 18

Ciliate diversity in cold water spring sources in Iceland

Ragnhildur Guðmundsdóttir[1,*], Agnes-Katharina Kreiling[1,2], Bjarni K. Kristjánsson[2], Viggó Þór Marteinsson[3,4], Snæbjörn Pálsson[1]

[1]Faculty of Life and Environmental Sciences, University of Iceland, 101 Reykjavík, Iceland
[2]Hólar University, 551 Sauðárkrókur, Iceland
[3]Matis ohf./Icelandic Food and Biotech R&D, Vínlandsleið 12, 113 Reykjavík, Iceland
[4]Faculty of Food Science and Nutrition, University of Iceland, 101 Reykjavík, Iceland

ABSTRACT: Cold groundwater springs at the edges of lava fields along the volcanic active zone in Iceland are an interesting habitat, presenting an ecotone between groundwater, surface water and the terrestrial ecosystems. They are categorized as fennoscandian mineral-rich springs according to the European Nature Information System (EUNIS) classification (C2.111 European Environmental Agency) and have a high conservation value. They are also an island-like phenomena in the landscape and, together with the stable chemical and physical properties of the groundwater, make excellent study sites for testing questions regarding community assembly theory. To explore the biota of these systems, we applied environmental metabarcoding to assess ciliate diversity in this habitat. DNA was extracted and metabarcoding based on the 18S rRNA gene was conducted for (1) water samples and (2) glass beads as support for biomass development. Alpha diversity for ciliate communities in the spring sources increased with temperature, and limnocrene springs had fewer, more abundant taxa than rheocrene springs. Differences were observed between the water samples and the glass bead samples, mainly in terms of abundance. When considering only the water samples, no variation was found among spring source communities, indicating that stochastic processes such as dispersal and ecological drift might be important in shaping the community composition.

KEY WORDS: Protozoa · Environmental DNA · Metabarcoding · Groundwater/surface water ecotone

1. INTRODUCTION

Iceland is rich in groundwater as a result of high precipitation (Árnason 1976, Koreimann et al. 1996) and large glaciers (Sveinbjörnsdóttir & Johnsen 1992). The amount of groundwater is not evenly distributed throughout the country. The area within the volcanic active zone along the tectonic boundary from the southwest to the northeast, with highly porous lava fields (Sigurdsson & Stefansson 2002), is generally richer in this important water resource than the older rock formations outside this zone. Springs are commonly found at the edges of the lava fields and are classified as fennoscandian mineral-rich springs (C2.111 European Environmental Agency). Being an endangered habitat, they have a high conservation value (Council Directive 92/43/EEC 1992, Council of Europe 2010). These spring sources are an ecotone between the surface waters, groundwater, and the terrestrial overlaying ecosystem (Scarsbrook et al. 2007). Spring sources have 2 sides: the surface waters and waters below ground (subsurface water). The surface water is exposed to sunlight and water flows relatively freely. In the subsurface water, sunlight is absent and the waterflow is controlled by sediments (Vervier et al. 1997)

*Corresponding author: rag41@hi.is

or by lava substrate as in the case of springs within the volcanic active zone of Iceland.

Cold groundwater spring sources are island-like structures in the landscape with stable environmental variables (van der Kamp 1995, Szczucińska & Wasielewski 2013). This makes springs an excellent study object for testing questions related to community assembly theory, e.g. whether the diversity patterns in community composition have mainly risen from neutrally driven processes such as geographical isolation, historical connections and demographics, or environmental factors (Teittinen & Soininen 2015, Power et al. 2018). Previous work has found that both ecological and geographical factors shape the diversity of invertebrates in cold springs in Iceland. In a study of invertebrate communities, the composition varied between spring types, where rheocrene springs (forming a stream) and limnocrene springs (forming a pool) were found to differ (Govoni et al. 2018, Kreiling et al. 2018). In Iceland, there are few studies on eukaryotic microbial ecology in general. Studies on ciliates in hot springs (Aguilera et al. 2010) and in streams across a geothermal gradient in the Hengill area, SW Iceland (Plebani et al. 2015), found that both temperature and substrate played a role in shaping the community composition for ciliate assemblies. Bacteria and fungi communities in cold springs in Iceland were found to differ mainly among geographical regions (Guðmundsdóttir et al. 2019). For ciliates it is an ongoing debate whether a geographical distribution pattern can be found or not (Foissner 2008, Fenchel et al. 2019). This can be tested in our spring source habitat with its island-like structure.

The endemic groundwater amphipod *Crangonyx islandicus* (Svavarsson & Kristjánsson 2006) was discovered in cold groundwater spring sources in Iceland within the volcanic active zone. The habitat of the amphipods is within the subsurface part of the spring sources, and they are not found in the aboveground counterpart. As groundwater ecosystems are usually not easily accessible; they are frequently studied at spring sources, their natural access points, when other access points such as caves and boreholes are not available (Simon et al. 2001, Farnleitner et al. 2005). The amphipods were originally discovered while studying 3 spine stickleback *Gasterosteus aculeatus* in lake Þingvallavatn (Kristjánsson et al. 2002) that included the spring sources within the lake, which initiated surveys across Iceland for various other taxa including Arctic charr *Salvelinus alpinus* (Kristjánsson et al. 2012) invertebrate communities in general (Govoni et al. 2018, Kreiling et al. 2018), Clitellata (Klinth et al. 2019), bacteria (Guðmundsdóttir et al. 2019) and fungi (Wurzbacher et al. 2020). A study of ciliates associated with *C. islandicus* revealed that at least 2 taxa of ciliates of the orders Apostomatida and Philasterida were associated with the amphipod (Gudmundsdóttir et al. 2018). Apostomatida is a specialized crustacean parasite, but the order Philasterida is more diverse in terms of lifestyles, ranging from free-living to facultative parasites (Lynn 2008).

In the present study we first assessed the diversity of ciliate protozoans in cold groundwater springs in Iceland which are either suspended in the water current or in a biofilm. This was done by applying DNA metabarcoding (Taberlet et al. 2012, 2018). Secondly, we inspected if and how environmental variables, spring type and geographic location shape the community composition in this habitat, and further related it to the ongoing debate on distribution patterns of microorganisms, i.e. whether 'everything is everywhere' (Beijerinck 1913, Baas-Becking 1934) or if geographical patterns in distribution can be detected for ciliate communities in spring sources. Thirdly, we assessed the ciliate diversity associated with *C. islandicus* using metabarcoding and compared it with a previous study of ciliates associated with the amphipod, in order to identify potential symbionts/parasites or food items in this peculiar system.

2. MATERIALS AND METHODS

2.1. Sampling and sample preparation of biological samples

2.1.1. Water samples

Water samples were collected in June, July and August in 2014 and 2015 at 26 spring sources (12 limnocrene springs and 14 rheocrene springs; Fig. 1, Table 1). In addition, samples in 16 springs were taken 2 m downstream of the source, which we refer to as surface samples. At each sampling site, 5 l of water were collected into bottles that had previously been washed with HCl (10%) and autoclaved. In 2014, water was obtained using a drill pump and a hose, whereas in 2015 it was collected directly into the bottles. The hose end was put approximately 5 to 10 cm or as far as possible into the spring source. Due to the structure of the lava in the spring sources, it was not possible to reach far into the subsurface groundwater system. The water samples were stored in a cooler for up to 24 h, and were then filtered through Sterivex^{TM} filters (GV Durapore, pore size

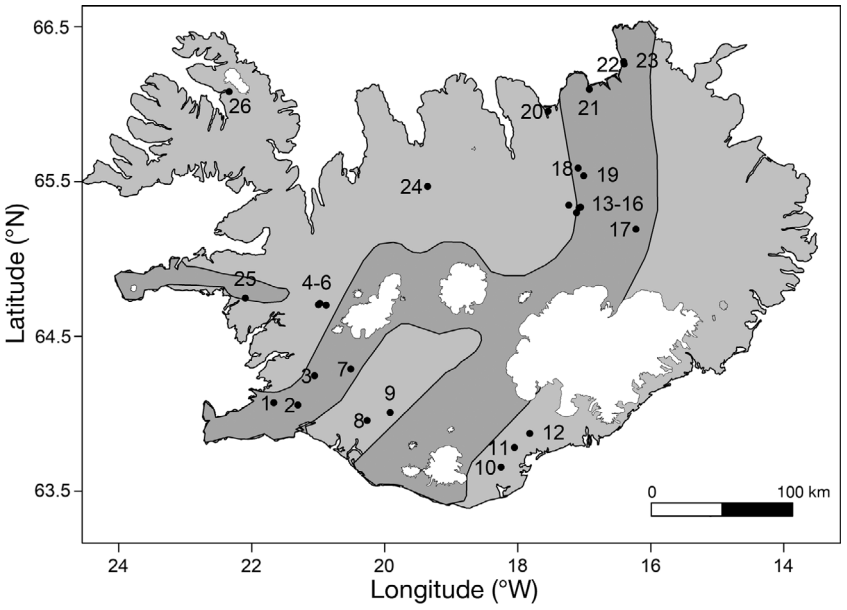

Fig. 1. Sampling locations (Sites 1–26) within Iceland in cold groundwater spring sources at edges of lava fields. Volcanic active zone is represented in dark grey and glaciers in white

0.22 µm cellulose membrane filter, Millipore Corporation). The filters were stored in the dark and frozen at −25°C until DNA extraction.

2.1.2. Glass bead samples

Glass beads, placed in net envelopes were incubated in 7 spring sources (Table 1) for 8 to 10 wk in the summer of 2014, serving as an artificial support to facilitate development of microbial biofilms and therefore attract protozoan grazers like ciliates. The envelopes had a mesh size of 1 mm, and included 120 beads, with a total surface area of 60.3 cm^2 and weight of 10 ± 0.1 g. After the *in situ* incubation period the glass bead nets were collected and stored wet in *in situ* water, in aluminium foil in a cooler in the dark until they were processed. The net envelopes were put in 30 ml of sterile water and shaken for 20 min before filtered through Sterivex™ filter (0.22 µm) as the water samples. The filters were stored in −25°C until the DNA extraction. See also Guðmundsdóttir et al. (2019).

2.1.3. Amphipod samples

Three specimens of *Crangonyx islandicus* were sampled in 2012 and 2013, at 3 geographically separated springs: Site 3 Southwest Iceland, and Sites 13 and 20 North Iceland (Fig. 1, Table 1). The specimens were collected by applying electro-fishing gear in the spring sources and catching them with dip nets as they floated out with the spring water. The specimens were stored in 96% ethanol and DNA extracted following the protocols described in Guðmundsdóttir et al. (2019). The 3 specimens were included in the metabarcoding analysis (see Section 2.3) despite low numbers and compared both to the water samples and to our previous results obtained with sanger sequencing and microscopy (Gudmundsdóttir et al. 2018).

2.2. Environmental variables

Environmental variables included in the analyses were altitude above sea level (m), temperature (°C), pH, oxygen saturation (%), presence of fish and amphipods, and substrate type (as predominately soft substrate, e.g. sand or mud, or as predominately hard substrate, e.g. lava rock) (Table 1). In addition, spring type (limnocrene or rheocrene), location of sample taken within each spring (hereafter referred to as source or surface), and latitude and longitude were included. Diversity indices for bacteria from a previous study (Guðmundsdóttir et al. 2019) were also included for comparison. One location was much warmer 42.6°C (Site 24, Northwest Iceland); this site was included for the overall comparison but omitted from statistical analysis. Oxygen saturation, temperature, and pH were measured with a multi-probe sonde (HYDROLAB DS5).

2.3. DNA extraction, amplification and sequencing

The total DNA was extracted from the Sterivex™ filters, and the filters were washed in a SET (sucrose, ethylenediamine tetraacetic acid, Tris-HCl) buffer and lysozyme solution, digesting the solution with Proteinase K (Neufeld et al. 2007). The DNA was purified with phenol-chloroform extraction using a rotating hybridization oven (Hybridiser HB-1D, Techne, Bibby Scientific). All the reagents for these steps were from Sigma-Aldrich, and the protocol used was based on Neufeld et al. (2007). The DNA from the amphipods was extracted directly from

Table 1. Sample information for environmental and geographical variables for a study on ciliate communities in spring sources in Iceland. Sample name consists of map number (as in Fig. 1), sample type (w: water, g: glass beads, a: amphipods), year (2012–2015) and source (s) or surface (o). Reads are raw read number from each sample. Lat N: latitude °N; Lon W: longitude °W; Alt: altitude above sea level (m); Temp: temperature (°C); Oxysat: oxygen saturation (%). Spring type are rheocrene (r) and limnocrene (l). Fish: presence of fish in the spring, where 0 indicates absence and 1 presence; *C.isl*: presence of *Crangonyx islandicus* in the spring, where 0 indicates absence and 1 presence; Substrate: type of benthic substrate, categorized into hard or soft. NA: data not available

Sample name	Reads	Lat N	Lon W	Alt	Temp	pH	Oxysat	Spring type	Fish	*C.isl*	Substrate
1.w.15.s	94 615	64.071	21.668	121	5.3	8.7	80.6	r	0	0	Hard
2.w.15.o	38 253	64.057	21.307	384	5.4	8.2	74.8	r	0	0	Hard
2.w.15.s	17 386	64.057	21.307	384	5.8	7.6	74.5	r	0	0	Hard
3.a.12.s	92 664	64.246	21.055	109	3.6	NA	NA	l	1	1	Hard
3.w.14.s	140 322	64.246	21.055	110	4.3	9.0	77.7	l	1	1	Hard
3.w.14.s	96 107	64.246	21.055	109	3.6	8.7	76.1	l	1	1	Hard
3.g.14.s	133 528	64.246	21.055	109	3.6	8.7	76.1	l	1	1	Hard
3.g.14.s	202 309	64.246	21.055	109	3.6	8.7	76.1	l	1	1	Hard
3.w.15.o	123 739	64.246	21.055	109	3.6	8.7	76.1	l	1	1	Hard
3.w.15.s	79 026	64.246	21.055	109	4.3	9.0	77.7	l	1	1	Hard
4.w.15.o	41 024	64.704	20.998	71	5.6	8.5	76.0	r	1	0	Soft
4.w.15.s	70 495	64.704	20.998	71	6.3	8.1	75.0	r	1	0	Soft
5.w.15.s	58 939	64.701	20.880	128	3.4	9.4	78.0	l	1	0	Hard
6.w.15.o	132 989	64.714	20.977	100	3.9	7.7	75.3	r	1	0	Hard
6.w.15.s	79 784	64.714	20.977	100	4.0	7.6	75.3	r	1	0	Hard
7.g.14.s	4933	64.290	20.512	184	2.4	9.3	78.1	l	0	1	Hard
7.w.14.s	190 130	64.290	20.512	184	2.4	9.3	78.1	l	0	1	Hard
7.g.14.s	301 959	64.290	20.512	184	2.4	9.3	78.1	l	0	1	Hard
7.w.15.o	33 666	64.290	20.512	184	2.4	9.3	78.1	l	0	1	Soft
7.w.15.s	22 714	64.290	20.512	184	3.4	9.4	80.2	l	0	1	Soft
8.w.15.s	125 131	63.957	20.265	78	5.5	7.9	75.6	r	1	1	Hard
9.g.14.s	6360	64.008	19.919	130	5.0	8.0	73.1	r	1	1	Hard
9.g.14.s	70 347	64.008	19.919	130	5.0	8.0	73.1	r	1	1	Hard
10.w.15.s	7584	63.655	18.252	33	7.5	7.9	77.8	l	1	0	Soft
11.g.14.s	29 422	63.782	18.050	30	5.5	NA	NA	r	0	1	Soft
11.g.14.s	45 052	63.782	18.050	30	5.5	NA	NA	r	0	1	Soft
11.w.14.s	173 396	63.782	18.050	30	5.5	NA	NA	r	0	1	Soft
12.w.15.s	69 084	63.873	17.820	53	5.1	7.5	76.1	l	1	0	Soft
13.w.15.o	22 164	65.331	17.078	430	5.9	8.7	69.1	r	0	0	Soft
13.w.15.s	45 726	65.331	17.078	430	8.6	8.8	69.5	r	0	0	Soft
14.w.15.o	87 879	65.299	17.119	437	4.1	8.9	62.6	r	0	0	Soft
14.w.15.s	55 141	65.299	17.119	437	4.5	9.0	66.5	r	0	0	Soft
15.a.12.s	115 140	65.336	17.232	382	3.7	NA	NA	r	0	1	Hard
15.g.14.s	221 394	65.336	17.232	382	3.7	9.0	70.8	l	0	1	Hard
15.g.14.s	28 733	65.336	17.232	382	3.7	9.0	70.8	l	0	1	Hard
15.w.15.o	122 805	65.336	17.232	382	3.7	9.0	70.8	r	0	1	Hard
16.w.15.o	6489	65.334	17.057	441	5.5	9.0	70.5	r	0	0	Hard
16.w.15.s	29 472	65.334	17.057	441	7.3	8.6	69.3	r	0	0	Hard
17.w.15.o	22 432	65.192	16.225	493	5.9	6.8	65.6	l	1	1	Soft
17.w.15.s	27 843	65.192	16.225	493	5.6	6.8	66.3	l	1	1	Soft
18.w.15.s	39 872	65.537	17.008	285	4.2	8.8	67.7	l	1	0	Hard
18.w.15.o	16 565	65.537	17.008	285	4.5	9.0	63.3	l	1	0	Hard
19.w.15.o	53 525	65.587	17.093	284	4.7	7.7	44.6	l	0	0	Hard
19.w.15.s	27 824	65.587	17.093	284	5.1	7.7	51.5	l	0	0	Hard
20.a.12.s	282 860	65.954	17.545	8	3.8	NA	NA	l	1	1	Hard
20.w.14.s	167 789	65.954	17.545	8	3.8	7.4	55.0	l	1	1	Hard
21.w.15.s	54 865	66.096	16.925	6	4.9	8.0	77.8	l	1	0	Hard
22.w.15.o	84 497	66.258	16.401	28	3.8	7.9	76.8	r	1	1	Soft
22.w.15.s	56 508	66.258	16.401	28	4.1	8.0	76.7	r	1	1	Soft
23.w.14.s	67 408	66.275	16.409	180	4.0	7.6	79.2	l	1	1	Hard
23.g.14.s	16 000	66.275	16.409	180	4.0	7.6	79.2	l	1	1	Hard
23.g.14.s	94 514	66.275	16.409	180	4.0	7.6	79.2	l	1	1	Hard
23.w.15.o	78 318	66.275	16.409	9	3.8	7.5	78.3	l	1	1	Soft
23.w.15.s	49 868	66.275	16.409	9	4.5	7.6	79.2	l	1	1	Soft
24.w.15.o	9141	65.469	19.357	62	40.2	8.5	86.3	r	0	0	Hard
24.w.15.s	28 913	65.469	19.357	62	42.6	8.5	80.1	r	0	0	Soft
25.w.15.o	13 884	64.748	22.097	62	4.6	5.3	79.2	r	1	0	Soft
26.g.14.s	102 667	66.080	22.340	NA	6.6	NA	NA	NA	0	0	NA

whole animals using 6% Chelex 100 (Bio-Rad Laboratories) and Proteinase K (Kornobis et al. 2010). PCR amplification of the DNA for all sample types (water, glass beads and amphipods) was done in a 25 µl reaction volume, containing 0.2 µM dNTPs, 1X Phusion HF buffer, 1 mM $MgCl_2$, with 0.1 mg ml^{-1} BSA, 0.1 µM each primer 0.01 U $µl^{-1}$ Phusion® High-Fidelity DNA polymerase (New England BioLabs) and 0.3 ng $µl^{-1}$ of template. The conditions for the PCR were 98°C 30 s for initial denaturing step and then 35 cycles of 98°C 10 s, 55°C 30 s, 72°C 30 s, with a final elongation step at 72°C for 7 min.

The primer pair used was CiliF and CiliR (Tapio et al. 2016), amplifying a 286 bp fragment of the V2-V3 region of the18S rRNA nuclear gene. *In silico* PCR performed with Obitools indicated that these primers identify 77% of ciliates at the order level and 70% at the family level as tested for the NCBI:taxid 5878 on the embl140 database allowing for 3 errors in forward and reverse primers. PCR products were purified and concentrated with illustra™ GFX™ PCR DNA and Gel band Purification Kit, followed by an indexing procedure using Nextera XT Index Kit, according to the manufacturer's instructions. The DNA fragment size was estimated using the Agilent High Sensitivity DNA kit (Agilent Technologies).

Library quantification, normalization, pooling and library denaturing followed the 16S rRNA gene Metagenomic Sequencing Library preparation protocol (Part # 15044223 Rev. B). DNA amplicon sequencing was performed on a MiSeq Illumina platform.

2.4. Sequencing data analysis

OBITools (Boyer et al. 2016) was used for raw sequence handling where the paired end reads were merged using a criterion of a minimum of 40 bp overlap. To reduce the influence of rare variants, sequences which were at least 200 bp length and were represented by 10 or more reads (as suggested by Brown et al. 2015) were kept for taxonomic assignment. To reduce computational time, sequences were dereplicated, and single representative sequence submitted for taxonomic classification and OTU clustering at 98% similarity, as recommended using the 128 release of the SILVAngs database (Quast et al. 2013, Yilmaz et al. 2014). As the hit can be divergent from the query sequence (e.g. >5% divergence) or the reported identity can be low due to partial BLAST alignments, the hits were accepted if average sequence identity and alignment coverage was larger than 93, a threshold which has been found empirically to give reliable results (SILVAngs user guide: www.arb-silva.de/ngs/service/file/?file=SILVAngs_User_Guide_15_12_15.pdf). The taxonomic units that resulted from this clustering criteria are referred to hereafter as operational taxonomic units or OTUs, and these are used for calculations of alpha and beta diversity. For eliminating chimeras, the program Decipher (Wright et al. 2012) was used. The SILVAngs output was prepared with an in-house script for analysis in R, version 3.5.2 (R Core Team 2017). All non-ciliate taxa were removed from the data and not analysed. The nucleotide sequence data reported are available in the GenBank databases under the BioProject accession number PRJNA623026.

2.5. Statistical analysis

Prior to the diversity assessment, the frequency observed for each OTU was rarefied to smallest number of reads obtained per sample (4933), using the rrarefy command in the vegan package (Oksanen et al. 2017) in R (R Core Team 2017). Five samples were removed from the dataset as they had fewer read numbers than 4933.

Alpha diversity was analysed visually with a bar plot for the most common groups of taxa (>2%). Three alpha diversity indices were calculated: richness or number of OTUs, Shannon diversity index and Shannon evenness using vegan (Oksanen et al. 2017) in R (R Core Team 2017). The Shannon diversity index is known to be a robust index for diversity assessments (Haegeman et al. 2013), and the Shannon evenness is here calculated as the ratio of the Shannon diversity index and the richness as in Borcard et al. (2018). Variation in the alpha diversity indices with respect to environmental variables, spring type and location was analysed with multiple regression following a stepwise model selection using the step function in the stats package in R (R Core Team 2017). Multicollinearity of environmental variables was eliminated in accordance with suggestions in Dormann et al. (2013), where correlation of $|r| > 0.7$ was used as a threshold for excluding variables and evaluated further with the variance inflation factor for the model, using the vif function in the car package (Fox & Weisberg 2019) in R. Correlation between ciliate diversity and bacteria diversity from the same springs (Guðmundsdóttir et al. 2019) was tested with Pearson's correlation.

Beta diversity of the spring community was summarized with Bray-Curtis (BC) distances and its relationship with environmental variables, spring type

and location tested with permutational analysis of variance (PERMANOVA) applying the adonis function in the vegan package (Oksanen et al. 2017) in R. The dissimilarities of the samples were summarized with principal coordinate analysis (PCoA) using both the packages vegan and ape (Paradis et al. 2004) in R. The function envfit from vegan was used to do the post projection of environmental variables onto the different axes in the PCoA plot. This was first done for both water samples and glass beads together and then for water samples from 2015 separately in order to keep balance in the study design, as the samples from 2014 were taken at the source whereas the samples from 2015 were both taken at the source and the surface.

The dependency of the pairwise BC distances between samples on geographic distances which may indicate an isolation by distance was tested separately for water and glass bead samples with Mantel tests using the ape package in R (Paradis et al. 2004). As the correlation summarized with the Pearson's correlation coefficient was nonsignificant, the beta diversity was summarized with the average of the diversities from the different samples with its standard deviation. This was done separately for the water and glass bead samples. To evaluate whether the 2 sample types differ with respect to species composition and in frequencies of the OTUs, the analysis was re-done by solely basing the BC distances on binary information (presence–absence data).

The overlap of ciliate taxa for all sample types was inspected using a Venn diagram drawn with the VennDiagram package (Chen 2016) in R. The taxa indicator analysis was done with Dufrene-Legendre Indicator Species Analysis using the indval function in the labdsv package in R 3.5.2 (Roberts 2019) where the samples had been split into temperature categories according to Tuxen's temperature classification of springs in Iceland as 'cold' (< annual mean air temperature), 'tepid' (> annual mean air temperature, < mean maximum air temperature) and 'hot' (> mean maximum air temperature) (Tuxen 1944, Kreiling et al. 2018).

3. RESULTS

3.1. Sequencing results and dominant taxa

The number of ciliate sequences obtained was 4 511 194 from 58 samples, collected at the 26 locations (Table 1). The total number of ciliate OTUs was 170. After rarefication there were 261 449 reads where the 2% most common taxa belong to 15 OTUs with 74.0% of the reads. The dominant taxa were Hymenostomatia (14.7%), followed by *Tetrahymena* (13.0%) and *Pseudochilodonopsis* (7.4%). These groups along with other 12 OTUs that belong to the 2% most common taxa are mostly free living and feed on bacteria and microalgae (Table 2). The exception of this is the genus *Fusiforma*, which is a crustacean symbiont (Chantangsi et al. 2013). *Fusiforma* was the single most common taxa found in the amphipod samples.

3.2. Ciliate diversity in springs

3.2.1. Alpha diversity

Ciliate diversity based on the Shannon diversity index increased with temperature (slope (b) = 1.51, 95% confidence interval = 0.73, 2.23, $p = 3.90 \times 10^{-4}$, Fig. S1 in the Supplement; www.int-res.com/articles/suppl/a084p191_supp.pdf), and a similarly positive (although non-significant) effect was found for species richness ($p = 0.06$, Table 3). The Shannon evenness index was higher for rheocrene than limnocrene springs (slope (b) = 0.25, 95% confidence interval = 0.04, 0.47, $p = 0.02$, Table 3, Fig. S2). Other environmental variables (substrate type, source or surface and geographical locations) were not associated with the diversity and evenness. Species richness was not influenced by any of the environmental and geographical variables (latitude, altitude) ($p > 0.05$). Ciliate diversity and evenness did not correlate with the bacteria diversity or evenness ($p > 0.05$) (Table S1 in the Supplement). The Dufrene-Legende Indicator Species Analysis did not reveal any taxa as indicator groups for different environmental factors such as temperature classes of the springs based on Tuxen s temperature classes for invertebrate communities (Tuxen 1944, Kreiling et al. 2018).

3.2.2. Beta diversity

The greatest difference in community composition was found between the sample types (Table 4A), as the glass beads harboured different communities than the water sample ($F_{(1,36)} = 2.81$, $p = 0.0003$). This difference was also reflected in the comparisons between the spring sources and the surface samples ($F_{(1,36)} = 2.38$, $p = 0.01$), as the glass beads were only sampled at the source this difference was analysed again by excluding the

Table 2. Ciliate taxa more frequent than 2% in all sample types in spring sources in Iceland. When amphipod samples are excluded from the analysis, the genus *Fusiforma* drops out of the list, and when 24.w.15.o and 24.w.15.s are excluded (~42°C), the *Hemiurosomoida* drops out of the list. Abb.: abbreviation for taxa, see Fig. 2. %: percent of total reads

Taxa	Abb.	%	Mode	Habitat	Food	Reference
CV1-2A-17	CV1	2.6	Free living, planktonic	Suboxic pond		Slapeta et al. (2005)
Cyrtophoria	Cyr	3.6	Free living	Biofilms on substrates	Bacteria, small algae	Lynn (2008)
Fusiforma	Fus	4.7	Symbionts, parasites	In/on crustaceans		Chantangsi et al. (2013)
Glaucoma	Gla	3.0	Free living	Freshwater	Microphagous, but several species carnivorous on other ciliates	Lynn (2008)
Haptoria	Hap	2.4	Free living	Marine, brackish, freshwater, terrestrial	Flagellates, ciliates, and other protists	Lynn (2008)
Hemiurosomoida	Hem	2.0	Free living	Dry sediment of hot springs		Singh & Kamra (2015)
Holosticha	Hol	5.5	Free living	Marine, freshwater and sediment	Bacteria, algae, and smaller protists, ciliates	Lynn (2008)
Hymenostomatia	Hym	14.7	Free living or in cyst	Most in freshwater	Bacteria with some carnivorous and some histophagous or parasitic	Lynn (2008)
Hypotrichia uncultured	Hyp	2.9	Predominantly free living	Freshwater, saltwater, soil and moss		Lynn (2008)
Paraurostyla	Par	2.1	Free living	Marine, freshwater, and terrestrial	Bacteria, microalgae, and smaller protists, but also ciliates and even smaller metazoan	Lynn (2008)
Pseudochilo-donopsis	Pse	7.4	Free living	Marine, freshwater, and terrestrial	Bacteria, microalga-eand parasitic	Lynn (2008)
Stokesia	Sto	2.8	Free living	Freshwater, typically planktonic	Flagellates and micro-algae, such as diatoms	Lynn (2008)
Tetrahymena	Tet	13.0	Free living	Freshwater ponds	Bacteria and organic matter	Lynn (2008)
Trithigmostoma	Tri	2.8	Free living	Marine, freshwater, and terrestrial	Bacteria, microalgae and parasitic	Lynn (2008)
Trochilia	Tro	4.6	Free living	Marine, freshwater	Bacteria and microalgae	Lynn (2008)

beads (Table 4B). Furthermore, the community composition is related to temperature ($F_{(1,36)}$ = 1.75, p = 0.04).

The effects of the environmental variables on the taxonomic composition was also valuated by their correlation with the axes of the ordination plot. For the first 2 axes (Fig. 2A) an association was observed with the sample type (r^2 = 0.21, p = 0.001) and with source and surface (r^2 = 0.12, p = 0.006). Hymenostomatia, *Pseudochilodonopsis*, *Cyrophoria* and *Glaucoma* were abundant in the glass beads compared to the water samples, while *Tetrahymena*, *Stokesia* and the ciliate group CV1-2A-17 were more commonly found in the water samples

Table 3. Alpha diversity indices within sites for ciliate communities in spring sources in Iceland. Significant predictor variable found with a stepwise selection procedure on multiple regression models, along with the estimate, standard error, *t*-value, p-value and the adjusted r^2

Alpha diversity index	Coefficients	Estimate	SE	*t*	p	r^2
Richness	Intercept	11.26	6.07	1.86	0.07	0.15
	Temperature	2.59	1.34	1.93	0.06	
	Substrate	6.70	3.55	1.89	0.06	
Shannon diversity	Intercept	−1.12	1.77	−0.63	0.53	0.31
	Sample type	1.89	1.21	1.56	0.13	
	Temperature	1.51	0.39	3.90	3.20×10^{-4}	
Shannon evenness	Intercept	4.98	4.23	1.18	0.24	0.18
	Latitude	−0.10	0.06	−1.52	0.13	
	Spring type	0.25	0.11	2.43	0.02	
	Altitude	5.94×10^{-4}	3.46×10^{-4}	1.72	0.09	

(Figs. 2A & 3). Groups that were equally found in the water samples and on the glass beads were Haptoria, *Holosticha*, *Trithigmostoma* and *Trochilia* (Fig. 3).

The third and the fourth axes (Fig. 2B) were correlated with oxygen saturation ($r^2 = 0.12$, $p = 0.041$) and temperature ($r^2 = 0.18$, $p = 0.007$). The taxa *Glaucoma* and *Holosticha* were more abundant in oxygen-rich and colder springs, whereas *Paraurostyla* and *Stokesia* were found at higher temperature (Fig. 2B). The results did not differ when the analysis was done separately for the source and the surface (not shown).

When the analysis was based solely on the water samples none of the variables tested contributed to the variation among the spring water communities (Table 4B). The BC distances among the ciliate assemblages were independent of the geographical distances between the sampling locations as tested with a Mantel test ($r = -0.04$, $p = 0.65$) with mean dissimilarity of 0.77 BC ± 0.01 SE (Fig. S3A); a BC distance of 0 represents communities that are identical, and it is 1 if the communities are completely different. The differences in community composition

Table 4. (A) Permutational analysis of variance for the ciliate community in cold spring sources in Iceland including glass beads. Warm spring (Site 24, ~42°C) was excluded from the analysis.. Oxysat: oxygen saturation (%); Spring type: limnocrene and rheocrene; Altitude: altitude above sea level (m); Fish: presence–absence of fish; Substrate: hard and soft; Sample type: water samples and glass bead samples. (B) Permutational analysis of variance for the ciliate community in cold spring sources in Iceland; only water samples from 2015

Source of variation	df	SS	MS	*F*	r^2	p
(A)						
Oxysat	1	0.32	0.32	1.11	0.02	0.33
Latitude °N	1	0.29	0.29	0.98	0.02	0.45
Temp (°C)	1	0.50	0.51	1.75	0.03	0.04
Spring type	1	0.28	0.31	1.07	0.02	0.35
Source/surface	1	0.66	0.69	2.38	0.05	0.01
Altitude	1	0.18	0.18	0.60	0.01	0.87
Fish	1	0.25	0.24	0.85	0.02	0.63
pH	1	0.31	0.30	1.05	0.02	0.38
Substrate	1	0.46	0.46	1.58	0.03	0.07
Sample type	1	0.82	0.82	2.81	0.06	3.00×10^{-3}
Residuals	36	10.60	0.29		0.72	
Total	46	14.60			1.00	
(B)						
Altitude	1	0.28	0.29	0.93	0.03	0.27
Source/surface	1	0.36	0.35	1.16	0.03	0.24
pH	1	0.24	0.24	0.79	0.02	0.21
Oxysat	1	0.27	0.27	0.89	0.03	0.27
Latitude °N	1	0.24	0.24	0.77	0.02	0.53
Temp (°C)	1	0.39	0.38	1.23	0.04	0.50
Spring type	1	0.25	0.25	0.81	0.02	0.68
Fish	1	0.20	0.20	0.64	0.02	0.65
Substrate	1	0.45	0.45	1.45	0.04	0.22
Residuals	24	7.40	0.31		0.74	
Total	33	10.08			1.00	

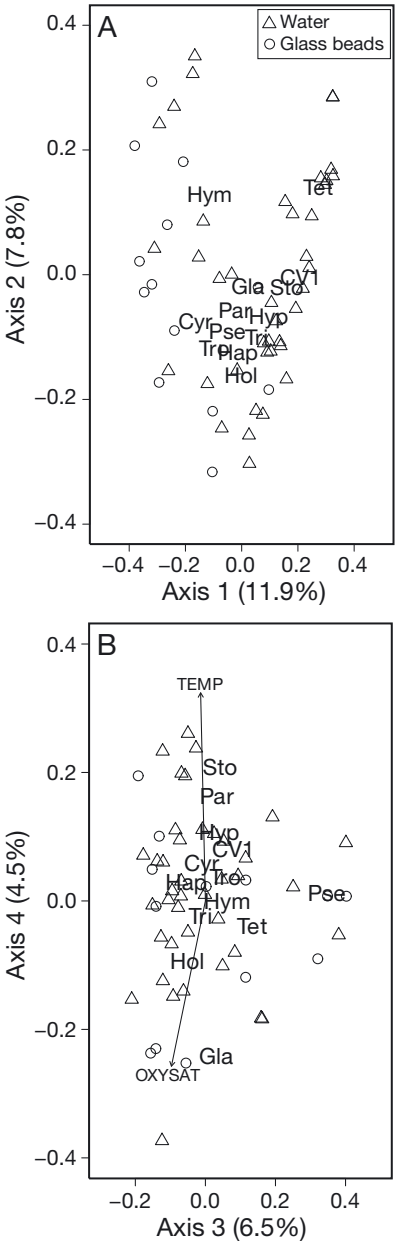

Fig. 2. Principal coordinate analysis (PCoA) plot based on Bray-Curtis distances for ciliate communities in spring sources in Iceland as obtained with water and glass bead samples. (A) Axes 1 and 2. (B) Axes 3 and 4. Together the first 4 axes explain 29.9% of the variance. The first axis splits the samples according to sample type (water and glass beads). CV1: CV1-2A-17; Cyr: Cyrtophoria; Fus: *Fusiforma*; Gla: *Glaucoma*; Hap: Haptoria; Hem: *Hemiurosomoida*; Hol: *Holosticha*; Hym: *Hymenostomatia*; Hyp: Hypotrichia uncultured; Par: *Paraurostyla*; Pse: *Pseudochilodonopsis*; Sto: *Stokesia*; Tet: *Tetrahymena*; Tri: *Trithigmostoma*; Tro: *Trochilia*; TEMP: temperature (°C); OXYSAT: oxygen saturation (%)

based on presence–absence (BC_{bin}) of taxa was also independent of geographic distances (r = −0.05, p = 0.53), but the average dissimilarity was lower (0.57 BC_{bin} ± 0.01 SE) (Table S2, Fig. S3B), indicating that the dissimilarity is boosted by frequency differences.

The glass bead community was independent of the geographical distances when considering both the abundances of taxa (0.69 BC ± 0.02, p = 0.65, r = 0.14) and presence–absence of taxa. It was similar but slightly lower to the value obtained for the water samples (0.59 BC_{bin} ± 0.01, p = 0.51, r = 0.09), Table S2 and Fig. S3.

3.3. Ciliates in amphipod samples

The composition of taxa was similar for all 3 amphipod specimens even though they came from sampling locations 300 km apart (Fig. 3). Only 12 ciliate OTUs were found in the amphipod samples (Table S3); 6 of them were unique to the amphipods, 4 were found both on glass beads and in the amphipod samples, and 2 were shared between all the sample types (Fig. 4). The OTUs found in both the amphipod samples and on the glass beads were the genus *Fusiforma* (Apostomatia), the genus *Miamiensis*, uncultured taxa from the subclass Peritrichia and unspecified OTU within the subclass Suctoria. *Fusiforma* was the most common ciliate taxon found with the amphipods after rarefication (90.0%) but was rarely found in the glass bead samples (0.04%) and never in the water samples.

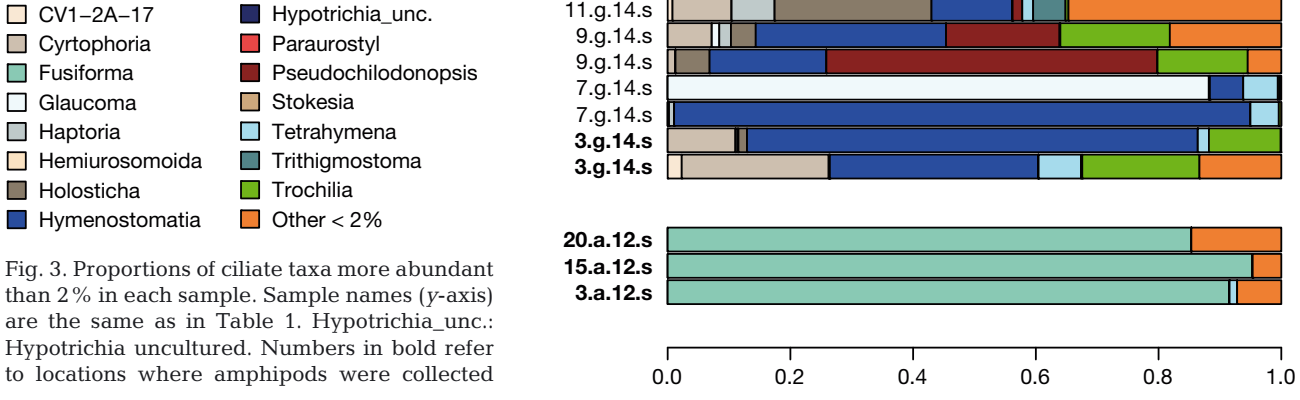

Fig. 3. Proportions of ciliate taxa more abundant than 2% in each sample. Sample names (*y*-axis) are the same as in Table 1. Hypotrichia_unc.: Hypotrichia uncultured. Numbers in bold refer to locations where amphipods were collected

Two OTUs were shared between all sample types: the genus *Tetrahymena* (water: 16.7%, glass beads: 3.0%, amphipods: 0.6%) and an uncultured taxon from the subclass Scuticociliatia (water: 0.02%, glass beads: 0.04%, amphipods: 0.6%). The 6 unique OTUs in the amphipod samples comprise an unidentified genus within the subclass Apostomatia, *Paramecium* (Peniculia), *Trichodina* (Peritrichia), *Paranophrys* (Scuticociliatia), *Hypocoma* (Rhynchodia), and *Euplotes* (Hypotrichia).

The similarity of the Apostoma sequences obtained here to *Crangonyx islandicus* type A (obtained in a previous study on ciliates associated with the *C. islandicus* (Gudmundsdóttir et al. 2018) ranged from 99 to 100% in most cases (Table 5), although variants were observed with 94 to 97% similarity. The *Miamiensis* sequences obtained here from the amphipod samples ranged from 97 to 99% similarity to the *C. islandicus* type P (Table 5), but a wider range of similarity (93 to 99%) was found in the environmental samples, indicating different species of this genus.

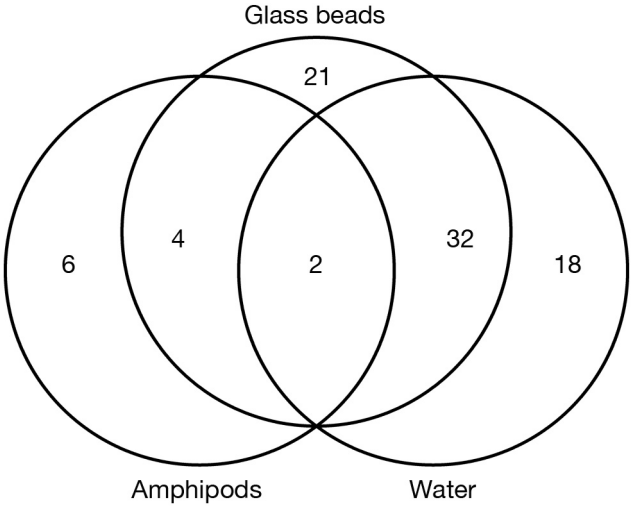

Fig. 4. Shared and unique taxa to each sample type at Sites 3, 15 and 20 (Lake Þingvallavatn, Lake Svartárvatn and Sandur, respectively; Fig. 1), where all sample types were collected

4. DISCUSSION

4.1. Ciliate diversity in the springs and environmental and geographical variables

The dominating ciliate taxa in the spring community obtained from both water and glass bead samples are mostly free living, non-sessile, and feeding on bacteria and small algae (Lynn 2008). They are known from various aquatic environments, both marine and freshwater. The only exception is the genus *Hemiurosomoida* that is known from dried sediment of a hot spring (Singh & Kamra 2015). It was common in the hottest spring in this study, Site 24 (Fig. 1) with a temperature of 42°C, although it was not exclusively found there.

The diversity of the ciliate communities, in the cold freshwater springs along the volcanic zone in Iceland, increases with increased temperature of the spring water. This trend is observed even within the small temperature range, but the diversity was otherwise independent of other environmental variables tested. Evenness showed a relationship with spring type, where limnocrene springs had fewer, more abundant taxa than the rheocrene springs.

The composition of communities differed between the sampling methods, as the glass beads incubated in the spring sources harboured a different community than the water samples taken at the spring source. When abundance is not considered but only presence–absence of taxa, there is no difference in the mean beta diversity between the 2 sample types. This means that the difference among water samples gets larger due to different proportions of taxa found

Table 5. BLAST queries for environmental sequences (bp length: 269–270) against Sanger sequences (bp length: 617–620) from Gudmundsdóttir et al. (2018). Location corresponds to numbers on map (Fig. 1), except for the sample obtained from *Crangonyx subterraneus* that was from the UK, and refers to where the type sequence was obtained from (in parentheses: locations where similar sequence variants were also obtained). Taxa: taxonomic identification according to the SILVAngs data base. Sim: similarity (%) match between Sanger sequence and eDNA sequence. Location eDNA: location where eDNA sequence was detected. Numbers refer to location on map (Fig. 1). Type: sample type; A: amphipods; G: glass beads; W: water

Query sequence	Location query	Taxa	Sim	Location eDNA	Type
Crangonyx_islandicus_ciliate_A.1	3 (8,15,20,23)	Fusiforma	99	3,7,9,15,20,23	A, G, W
Crangonyx_islandicus_ciliate_A.2	3 (8,15,20,23)	Fusiforma	99,100	3,7,9,15,20,23	A, G, W
Crangonyx_islandicus_ciliate_A.3	3 (8,15,20,23)	Fusiforma	99	3,7,9,15,20,23	A, G, W
Crangonyx_islandicus_ciliate_A.4	23 (3,8,15,20)	Fusiforma	99	3,7,9,15,20,23	A, G, W
Crangonyx_islandicus_ciliate_P	3,20	Miamiensis	99	3,20,25	A, G, W
Crangonyx_subterraneus_ciliate	Hertfordshire, UK	Miamiensis	98	3,20,25	A, G, W

at different sites, but these frequencies seem to be more stable in the glass beads. Previous work has shown that the process behind the biofilm formation is driven by a complex procedure involving species sorting for bacteria (Besemer et al. 2012) and that different incubational material may result in differences in communities based on factors such as roughness of the surface of the artificial support that is used (Voisin et al. 2016). Species sorting is also important for microbial eukaryotes like ciliates (Price & Morin 2004), and that might be the process behind higher abundance for some of the taxa in this study like Hymenostomatia and *Pseudochilodonopsis* that are abundant in most of the glass bead samples. Both members of the subclass Hymenostomatia and the genus *Pseudochilodonopsis* have diverse lifestyles ranging from bacterivorous to carnivorous and even histophagous parasites (Lynn 2008).

The differences between communities, tested separately for the water samples and the glass beads, were independent of the environmental variables and the geographical distances. This is interesting, considering that the distance between neighbouring springs ranges from 1 km between the closest springs to 300 km between those furthest apart. As the composition of taxa does not change with increased distance and is not explained by the variables tested in this study, the observed variation might be rooted in historical explanations such as species sorting and succession as well as environmental variables not measured in this study. Other factors that might be shaping the community are stochastic processes of ecological drift, i.e. due to random chances in species abundances and composition (Zhou & Ning 2017). The ciliates show different patterns from what we found for bacteria in the same springs, where the different geographical areas were found to differ in community composition (Guðmundsdóttir et al. 2019). In both cases stochastic processes such as dispersal and drift might be more important than deterministic processes like speciation and diversification if these microorganisms distribute easily among these habitats, but further studies are needed to disentangle these factors. This could explain why factors such as the source or surface at each spring site did not explain any of the variation in the beta diversity, although found to differ for fungi (Wurzbacher et al. 2020). In a study on ciliate assemblages based on morphological identification, in streams in Hengill, Iceland, the benthic substrate of the stream was found to play a role for the community composition of ciliates (Plebani et al. 2015). Sampling on the substrate might favour sessile taxa, whereas in the present study, the whole community (given the primer bias of amplifying only 77% at the order level and 70% at the family level) was collected and both sessile and mobile taxa were targeted. It would be interesting to look further into how the sessile taxa are represented in the dataset, but they are at least not among the most dominant taxa. Taxa such as *Vorticella* and *Stentor*, for example, are both represented in the glass bead samples and in the water samples but in low numbers.

4.2. Amphipods and ciliates

The diversity of the ciliate community found in the amphipod samples was much lower than for the other sample types. The amphipods are inhabiting the groundwater habitat with potentially low biodiversity, and the associated ciliate community is probably reflecting this. In richer systems, such as surface freshwater and marine environments, the epibiont community is usually more vivid with more species involved (Threlkeld & Willey 1993, Ólafsdóttir & Svavarsson 2002, Fernandez-Leborans & Von Rintelen 2007).

The 2 dominating ciliate OTUs found in the amphipod samples were apostomes, known to be exuviotrophic epibionts on crustaceans (Chatton & Lwoff 1935) and were closely related to the genus *Fusiforma,* and Philasterida from the genus *Miamiensis*. The same 2 groups were obtained in our previous study on ciliates associated with *Crangonyx islandicus*, type A and P (Gudmundsdóttir et al. 2018). Ten other ciliate OTUs were observed in low frequency in the amphipod samples. Of the 6 taxa that are found uniquely in the amphipod samples, 4 are parasitic (Lynn 2008); Apostomatia, *Trichodina*, *Paranoprhys* and *Hypocoma*. *Trichodina* and *Hypocoma* and all known members of the subclass Apostomatia are parasites or symbionts (Lynn 2008), and *Paranophrys* is facultative parasitic (Lynn 2008). Finding these in addition to the taxa already identified means that the parasite load of the amphipod is more than previously recorded. Only 2 taxa, *Paramecium* and *Euplotes,* unique to the amphipods are free living and feeding on bacteria and microalgae (Lynn 2008). If these 2 are food items for the amphipods, their low abundance might explain why they are not found in the environmental samples, or they may have a patchy distribution within the groundwater habitat. In addition to the aforementioned *Fusiforma*, Peritrichia and Suctoria were also found both in the amphipods and on the glass beads. These are sub-

classes of sessile ciliates that include both free-living groups and ectosymbiotic ones (Lynn 2008). As only 3 amphipod samples were successfully sequenced in this study, the results should be interpreted with caution.

In conclusion, the diversity of the ciliate communities in the cold water springs seems to be affected by temperature, despite the narrow range, and by neutral processes where long-distance dispersal and temporal fluctuations may dominate the possible associations with environmental factors. If dispersal is limited, we would expect some taxa to be abundant at some sites and lower in others, but as there was no association with geographical distances, our findings suggest that dispersal between spring sites is not limited for this group of organisms. A higher taxonomic resolution, targeting more variable genetic markers and all known ciliate taxa could have revealed more fine-scaled patterns. In addition, processes such as species sorting and succession, as well as environmental variables not measured in this study, may have contributed to the community patterns. Applying the metabarcoding on the groundwater amphipod *C. islandicus* confirmed previous findings of associated ciliate taxa but added 4 taxa of parasitic or symbiotic ciliates. The low number of ciliate taxa in the amphipod samples compared to the water samples from the spring source indicates that they may be in symbiotic relationship with the amphipods. The subsurface part where the amphipods are feeding is also less species rich than the surface waters of the springs, possibly reflecting its geologically isolated habitat.

Acknowledgements. This work was supported by the Icelandic Research Council (grant numbers: 130244-051 and 141863-051) and by the doctoral fund at the University of Iceland. We thank Eyjólfur Reynisson for guidance through sequencing, Stephen Knobloch and Cecile Le Sausse at Matís for lab assistance. We thank Vésteinn Snæbjörnsson for assistance with bioinformatics. We also thank the landowners and the director of the national park at Þingvellir for permission to sample.

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Editorial responsibility: Urania Christaki, Wimereux, France

Submitted: January 14, 2020; Accepted: May 5, 2020
Proofs received from author(s): June 11, 2020