Variation of *Legionella* spp. with Lake Depth and Season in Two Norwegian Drinking Water Sources

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ABSTRACT: In Norway, placement of the water treatment plant intake within the lake hypolimnion is considered a hygienic barrier against pathogens of fecal origin. It is unclear, however, whether this practice provides a barrier against opportunistic pathogens such as *Legionella*. In this study, water samples were collected at 10 m depth intervals near the drinking water intakes of two lakes. *Legionella* and one of their common hosts, *Acanthamoeba* spp., were quantified using culture-based assays (*Legionella pneumophila* only) and real-time quantitative PCR (qPCR). *L. pneumophila* and *Acanthamoeba* spp. were never detected by qPCR; *Legionella* spp., however, were present in all samples at concentrations ranging from 2.33 to 4.14 $\log_{10}[\text{copies/L}]$ in lake A and from 2.69 to 4.27 $\log_{10}[\text{copies/L}]$ in lake B. For most sampling months in both lakes, there was no significant difference between total bacteria and *Legionella* spp. concentrations at the intake depth versus those on the lake surface. The results of this limited investigation of two Norwegian water supplies suggest that placement of water treatment plant intakes within the hypolimnion may not afford a sufficient hygienic barrier against *Legionella*.

KEYWORDS: opportunistic pathogens, Legionella, thermocline, drinking water sources, hygienic barrier, amoebas
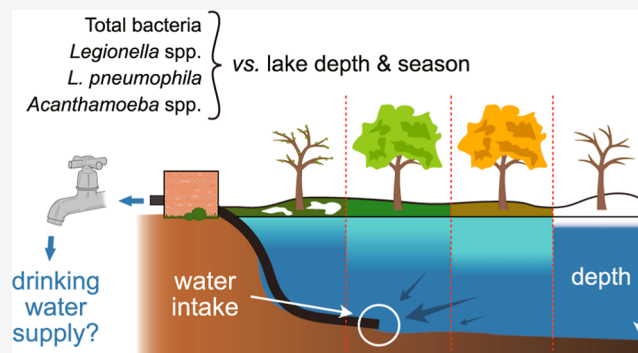
■ INTRODUCTION

Hygienic barriers are natural or constructed obstacles or actions that sufficiently remove or inactivate pathogens in order to mitigate waterborne diseases.[1] These barriers may limit pathogens from reaching the drinking water system intake, preventing pathogens from leaving the treatment plant or preventing contamination during water distribution to consumers.[2] Historically, Norwegian authorities required at least two independent hygienic barriers, each from a distinct prevention class.[2] Primary disinfection, commonly via chlorination or ultraviolet (UV) irradiation in Norway, is perhaps the most important barrier employed in many countries around the world. Characteristics of the drainage basin (i.e., land use) and the water source (e.g., water intake depth), however, are also deemed to be important in Norway.[2−4] Approximately 90% of Norway's inhabitants receive drinking water from surface sources, primarily freshwater lakes.[5]

Some chemical and microbiological contaminants may correspond to lake depth, particularly when a lake is thermally stratified and the contamination originates from a point source (e.g., wastewater discharge).[6−10] For example, concentrations of fecal indicator bacteria may differ by as much as 1 to 2 log between lake epilimnion and hypolimnion.[6,7,9] In contrast to fecal contaminants, however, it is unclear whether a similar depth dependency—and by extension, a hygienic barrier—exists for opportunistic pathogens found naturally and

ubiquitously in soils and surface waters, like *Legionella pneumophila* or other pathogenic *Legionella* spp. *Legionella* is a genus of more than 50 species that may intracellularly replicate inside eukaryotic hosts (e.g., *Acanthamoeba* spp.) and grow optimally in the range of 20 to 45 °C.[11] Upon exposure, the incidental infection of alveolar macrophage cells in humans may lead to influenza-like Pontiac fever or severe Legionnaires' disease, a potentially fatal pneumonia.[12,13] Although *L. pneumophila* is most commonly associated with clinical illness, at least 20 other species have been associated with Legionellosis.[14]
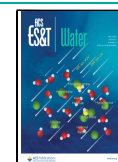
Legionella have been widely observed in lake water at various depths and in lake sediments,[15−21] but the abundance and diversity of *Legionella* spp. versus lake depth and across seasons are not well understood. More broadly, however, variations in lake microbiomes have been associated with the depth profiles of water temperature, dissolved oxygen, and other physical/chemical parameters.[22−27] Although *Legionella* may proliferate to hazardous concentrations far from the original water source—e.g., in premise plumbing[28−30]—the indigenous micro-

biome of the source water is nonetheless the origin of many taxa in the taps of end users.[31−34] Furthermore, the use of surface water for drinking water production—distributed without disinfectant—has been previously associated with Legionnaires' disease incidence in the Netherlands.[35] Infection incidence has also been linked to environmental factors like water temperature, rainfall, humidity, and watershed hydrology.[36−39]

The main objective of this study was to investigate seasonal variation in the concentrations of total bacteria, *Legionella* spp., *L. pneumophila*, and *Acanthamoeba* spp., in two Norwegian lakes that provide drinking water to the same municipality. Previous work hinted at regional differences in *Legionella* spp. and *L. pneumophila* concentrations and the microbiome of tap water within that city.[40,41] Because some areas of the municipal drinking water network receive water derived from only one of the lakes, there was interest in whether these regional differences in the microbiomes and *Legionella* concentrations were attributable to differences in the source water microbiomes, especially at the water intake depths.

■ MATERIALS AND METHODS

Site Description. Two lakes were investigated, which act as primary (lake A) and secondary/reserve (lake B) drinking water supplies to a Norwegian municipality. The lakes are situated 30 km apart and experience similar climates. Drinking water treatment plants receive raw water from lakes A and B via intakes at depths of 50 m and 30 m, respectively. Additional lake characteristics are summarized in Table S1 in the Supporting Information (SI). Notably, certain land use restrictions, together with the lake intake depths, act as the first hygienic barrier.

For lake A, water treatment includes intake screening (0.5 mm mesh), carbonation with CO_2, filtration-like passage through 3.7 m granular marble (primarily to harden the water for corrosion control but affecting some particle filtration), and then disinfection with NaOCl (dosed variably to achieve a target concentration of 0.05 mg/L as Cl_2 after a 30 min contact period) and UV irradiation (40 mJ/cm^2). At lake B, there is a 0.25 mm mesh screen at the intake and water is disinfected with UV and NaOCl, the same as for lake A. The disinfection steps act as the second hygienic barrier for both water sources, and there is little or no disinfectant residual in the distribution network.

Sample Collection and Storage. Water samples were collected near both water intakes from August 2019 to August 2020, using a Ruttner sampler at 10 m depth intervals (0 to 60 m for lake A and 0 to 30 m for lake B). From November 2019 until March 2020 (lake A) or until April 2020 (lake B), raw water samples were collected only at the treatment plants due to unsafe boating conditions; these were considered equivalent to lake water at the relevant depths for the intakes, although the water travels through a tunnel 4 km (lake A) or 1.4 km (lake B) prior to arriving at the treatment inlets. Monthly sample collection was typically carried out on two consecutive days—one lake per day. During all sample collection events, water was collected for analysis of pH, conductivity, turbidity, total organic carbon (TOC), dissolved organic carbon (DOC), and culturable *L. pneumophila*. In August 2019, October 2019, May 2019, and June 2020 (representing the summer, autumn, spring, and summer seasons, respectively), samples were also collected for DNA extraction. A summary of all sample collection events and the applicable water quality parameters is provided in the Supporting Information (Table S2).

For DNA analysis, water from each depth was transferred from the Ruttner sampler into sterile 2041 mL Whirl-Pak Stand Up Bags (Nasco; Fort Atkinson, Wisconsin; approximately 2 × 1.5 L per depth). For culturable *L. pneumophila*, water from the Ruttner sampler was transferred to one to three 120 mL sample bottles (IDEXX Laboratories; Westbrook, Maine). The pH, conductivity, and turbidity were measured either from the Whirl-Pak samples used for DNA extraction or from water collected in plastic 50 mL screw-cap, flat-bottom centrifuge tubes. Water samples for analysis of TOC and DOC were transferred directly from the Ruttner sampler to organic carbon-free 40 mL glass vials. Samples were transported on ice in coolers and processed, acid-preserved, or analyzed in the laboratory on the same day.

Triplicate 850 mL subsamples from the Whirl-Paks at each lake depth were individually vacuum-filtered through S-Pak mixed cellulose ester membrane filters to isolate the microbial biomass (0.22 μm pore size and 47 mm diameter; MilliporeSigma; Burlington, Massachusetts). Using ethanol-flamed forceps and micro-dissecting scissors, filter membranes were cut into approximately 5 mm squares, placed into ZR BashingBead lysis tubes containing 1000 μL of DNA/RNA shield (Zymo Research Corp.; Irvine, California), and stored at −20 °C until DNA extraction. Each sample batch was accompanied by a negative control, prepared by vacuum filtering 850 mL of autoclave-sterilized MilliQ water (121 °C for 15 min).

Water Quality. At each 10 m depth, temperature was measured using a glass thermometer affixed within the Ruttner sampler and dissolved oxygen (DO) was measured with an optical digital DO probe (Aqualabo; Marne, France). At the laboratory, pH and conductivity were measured using appropriate electrodes. Turbidity was measured using a 2100Q portable turbidimeter (Hach Company; Loveland, Colorado). TOC and DOC were measured by combustion (Standard Method 5310B) using an Apollo 9000 TOC analyzer (Teledyne Tekmar; Mason, Ohio). DOC samples were filtered using 0.45 μm nitrocellulose membranes, pre-rinsed with 200 mL of MilliQ water.

Estimating Thermocline Depths. Thermoclines were estimated from field temperature measurements using the split-and-merge algorithm,[42] implemented using the "wtr.layer" function in the R software package, *rLakeAnalyzer*.[43] Due to the ambiguous boundaries of the metalimnion, the lakes were divided into two strata for comparison purposes: an upper stratum from the lake surface (0 m) down to the estimated thermocline plus 5 m (i.e., both the epilimnion and an arbitrary "metalimnion") and a lower stratum or hypolimnion from this lower boundary down to the lake bottom. When no thermocline was detected, the lake was considered destratified.

DNA Extraction. Frozen filter membranes (in DNA/RNA shield) were thawed gradually on ice. Cell lysis was achieved using a Precellys Evolution homogenizer (Bertin Instruments; Paris, France), with the following program (recommended by Zymo Research): 1 min at 9000 RPM followed by 2 min rest, subsequently repeated three times. DNA was then isolated and purified using a Zymobiomics DNA/RNA Miniprep kit (Zymo Research), following the manufacturer's protocol. DNA extracts (100 μL) were stored at −20 °C until subsequent analysis.

Real-Time Quantitative Polymerase Chain Reaction (qPCR). Genetic targets of specific taxa were quantified by qPCR: total bacteria via a fragment of the 16S rRNA gene (primers 341F and 534R),[44] genus *Legionella* via the *ssrA* gene (primers PanLegF and PanLegR and probe PanLegP),[45] *L. pneumophila* by targeting its *mip* gene (primers LpF and LpR
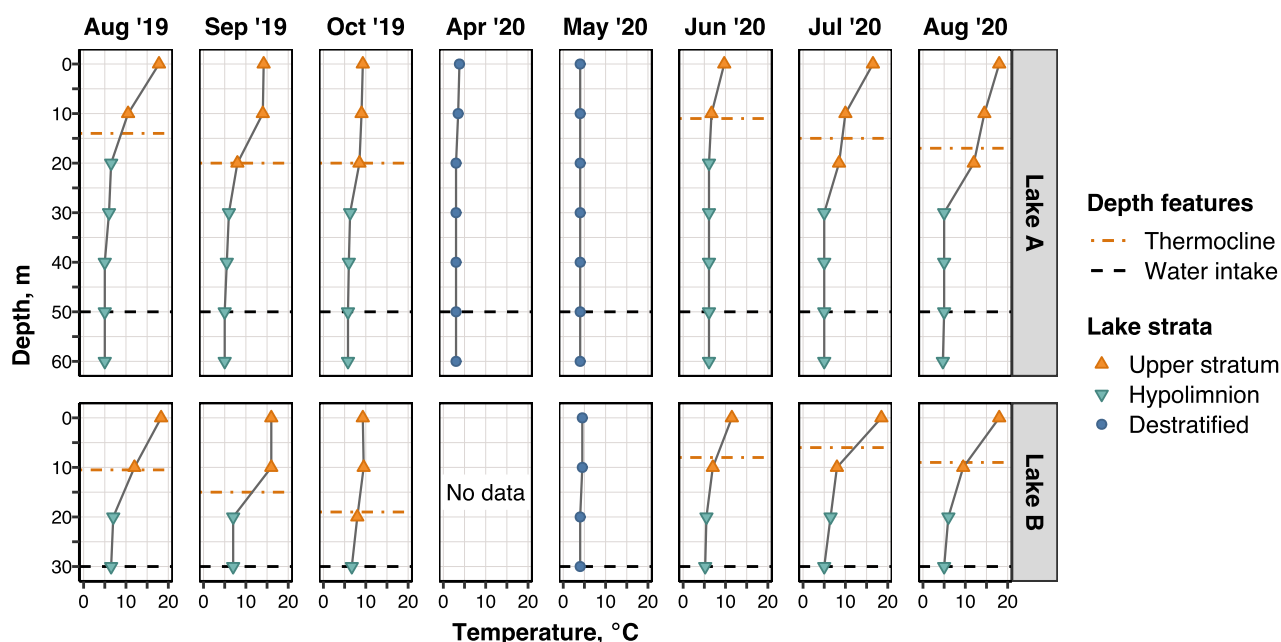
Figure 1. Monthly water temperature versus lake depth in Norwegian lakes A and B.

and probe LpP),[45] and genus *Acanthamoeba* via a fragment of their 18S rRNA gene (primers AcantF900 and AcantR1100 and probe Acant1000P).[46] All reactions were performed with a CFX Connect real-time PCR detection system (Bio-Rad Laboratories, Inc.; Hercules, California) and consisted of 10.0 μL of either Bio-Rad SsoFast EvaGreen Supermix with low ROX (16S rRNA genes) or Bio-Rad SsoAdvanced Universal Probes Supermix (*ssrA*, *mip*, and 18S rRNA genes), 20 μg of bovine serum albumin (Roche Diagnostics; Indianapolis, Indiana), primers, and probes to achieve the designated final concentrations, 2.0 μL of DNA extract, and PCR-grade water (Sigma-Aldrich; St. Louis, Missouri) for final reaction volume 20.0 μL. PCR conditions, primer/probe sequences, and final concentrations are summarized in Table S3.

Reference genes for each of the genetic targets (Table S4) were synthesized as gBlocks gene fragments (Integrated DNA Technologies, Inc.; Skokie, Illinois). These were serially diluted to produce standard curves during PCR, as summarized in Table S5. For 16S rRNA genes, a limit of quantification (LOQ) was defined as 10 times the gene copy number in PCR no-template controls (equal to 2.90×10^3 copies per reaction).[40] For *ssrA*, *mip*, and 18S rRNA genes, the LOQ was operationally defined as the lowest concentration in the standard curve or 10, 10, and 1.00×10^3 copies per reaction, respectively.

Culturable *L. pneumophila*. Culturable *L. pneumophila* were detected and enumerated via a Legiolert/Quanti-Tray system for non-potable water (IDEXX), as per the manufacturer's 1.0 mL protocol.[47] Humidity was maintained during incubation (7 days at 37 °C) to avoid moisture loss within the Quanti-Trays, as previously recommended;[48] average moisture loss was approximately 4.6 ± 2.5%. During each sample collection event, a blank control was included containing autoclave-sterilized MilliQ water (121 °C for 15 min). *L. pneumophila* and *Enterococcus faecalis* cultures were used as positive and negative controls, respectively, as per the recommendation of IDEXX.

Statistical Treatment and Hypothesis Testing. Hypotheses were tested (α = 0.05) using various statistical tools in R

software,[47] with support from *lme4*,[49] *emmeans*,[50] *car*,[51] and the *tidyverse*[52] packages.

Water Quality. We hypothesized that water temperature, DO, pH, conductivity, turbidity, TOC, and DOC may correlate with lake depth and may therefore correlate with one another. Spearman's rank correlation test was used for all combinations of the water quality parameters and lake depth; this analysis was limited to lake A due to insufficient data for lake B (only four discrete depths). Within each lake, water quality at the lake surface (depth = 0 m) was also compared to water quality at the water intake depth via paired *t* tests (observations paired with sample collection event). Finally, water quality was contrasted between the two lakes with paired *t* tests, pairing observations by the lake depth and collection event (thus excluding depths 40 to 60 and the April 2020 samples from lake A, for which lake B lacked corresponding observations).

Genetic Target Concentrations. To contrast mean target concentrations between the two lakes, mixed-effects linear models were fit via the *lme4* package.[49] These models attempted to explain the target concentrations using lakes (fixed effect) and with sample collection event and lake depth as random effects. From the models, the estimated marginal means were determined via the *emmeans* package,[50] and we assessed whether the mean target concentrations differed between lakes. Next, target concentrations at the lake surfaces were contrasted against concentrations at the respective drinking water intakes using paired *t* tests. The rationale was that the water at the intakes was the most direct consequence of the municipal water supply, while water at the surface was, we hypothesized, potentially the most different from that at the intake. Finally, as a broader investigation of lake ecology, simple or multiple linear regression was used to further elucidate whether lake depth and/or thermal stratification explained target concentrations within each lake during each of the four seasons. Four linear models were assessed for their explanatory power: target concentration predicted by (1) lake depth, (2) the thermal stratification layer, (3) lake depth and the thermal layer (terms added consecutively), or (4) lake depth and the thermal layer, with an interaction term (i.e., independent effects of lake depth

Table 1. Water Quality at the Lake Surfaces Versus at Their Respective Drinking Water Intakes[a]

site	surface [A]			intake [B]			diff. [A − B]			paired t-test			
	mean	±	SD	mean	±	SD	mean	±	CI	df	t stat.	p value	
Temperature, °C													
lake A	11.7	±	5.8	4.9	±	1.0	6.8	±	4.5	7	3.55	0.009	**
lake B	13.7	±	5.4	5.6	±	1.1	8.1	±	4.8	6	4.16	0.006	**
Dissolved Oxygen, mg/L													
lake A	10.1	±	1.6	10.6	±	1.6	−0.5	±	0.5	5	−2.45	0.058	.
lake B	9.8	±	1.9	10.5	±	1.3	−0.7	±	0.9	4	−2.04	0.110	
pH													
lake A	7.34	±	0.18	7.16	±	0.17	0.19	±	0.16	7	2.85	0.025	*
lake B	7.79	±	0.27	7.51	±	0.11	0.28	±	0.27	6	2.58	0.041	*
Conductivity, μS/cm													
lake A	58.9	±	4.7	56.9	±	1.5	2.0	±	4.1	7	1.16	0.286	
lake B	95.5	±	5.3	93.2	±	1.2	2.3	±	4.2	6	1.32	0.235	
Turbidity, FNU													
lake A	0.48	±	0.15	0.47	±	0.24	0.01	±	0.25	7	0.07	0.946	
lake B	0.46	±	0.30	0.43	±	0.22	0.02	±	0.27	6	0.21	0.842	
Total Organic Carbon (TOC), mg/L													
lake A	3.18	±	0.14	3.01	±	0.12	0.17	±	0.17	7	2.40	0.047	*
lake B	2.11	±	0.14	2.03	±	0.04	0.08	±	0.10	6	1.86	0.112	
Dissolved Organic Carbon (DOC), mg/L													
lake A	3.04	±	0.14	2.93	±	0.13	0.11	±	0.10	7	2.59	0.036	*
lake B	2.00	±	0.09	1.95	±	0.09	0.04	±	0.08	6	1.39	0.214	

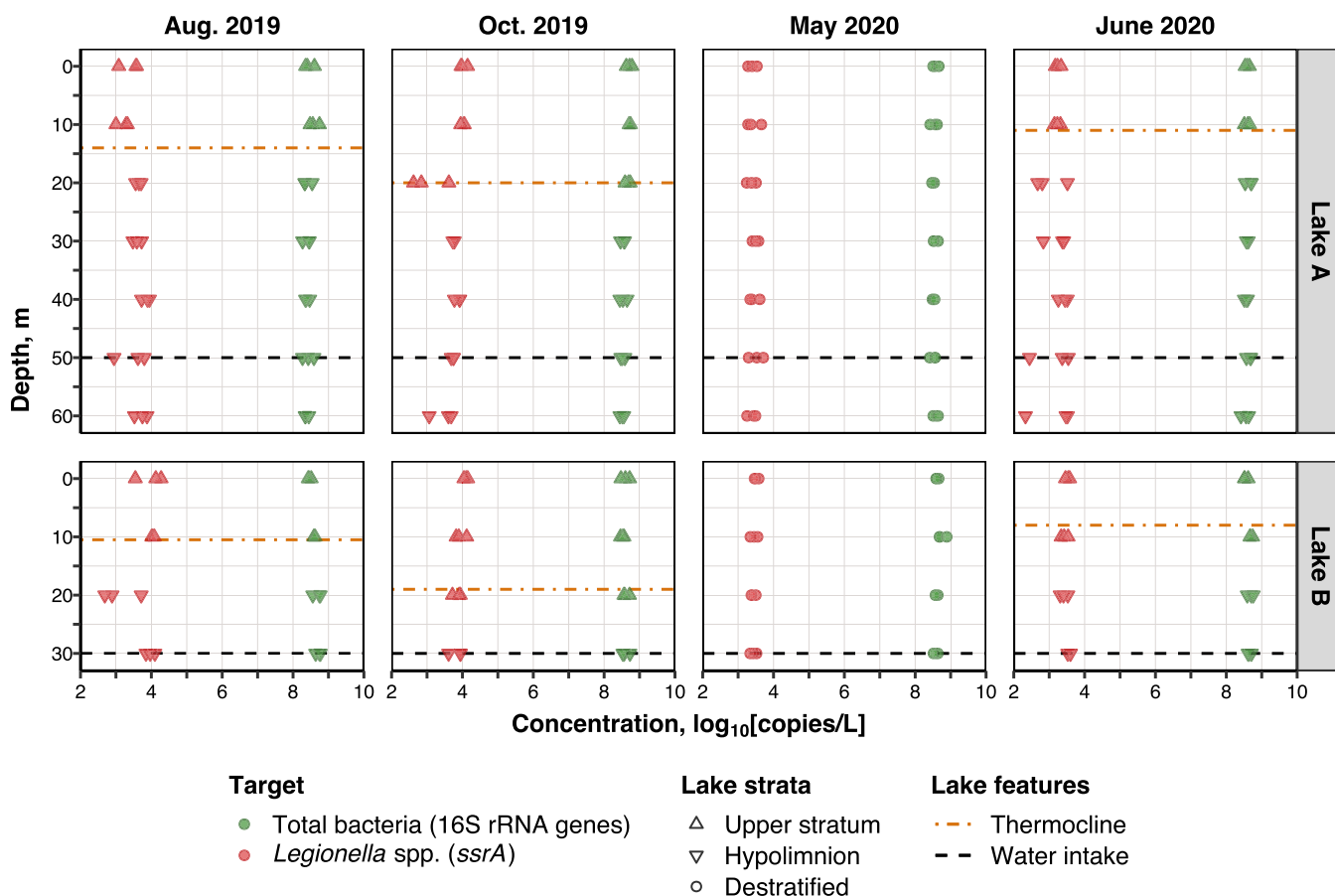[a]Significance codes for p: "***" ≤0.001; "**" ≤0.01; "*" ≤0.05; "." ≤0.1.

Figure 2. Genetic target concentrations of total bacteria and *Legionella* spp. (via qPCR) vs lake depth in Norwegian lakes A and B.

within each thermal layer). Linear models and model selection are described more thoroughly in the Supporting Information Materials and Methods (notably, the adjusted R^2). Selection was based primarily on whether models had a significant regression p value, followed by the lowest Akaike information criterion

Table 2. Genetic Target Concentrations (as $\log_{10}[\text{copies/L}]$) at the Lake Surface Versus at Drinking Water Intake[a]

target	event	surface [A]			intake [B]			diff. [B − A]			*t*-test			
		mean	±	SD	mean	±	SD	estimate	±	CI	df	*t* stat.	*p* value	
							Lake A							
total bacteria (16S rRNA genes)	Aug. 2019	8.45	±	0.13	8.43	±	0.16	−0.02	±	0.33	4	−0.21	0.846	
	Oct. 2019	8.71	±	0.08	8.52	±	0.06	−0.19	±	0.16	4	−3.25	0.031	*
	May 2020	8.58	±	0.08	8.51	±	0.09	−0.06	±	0.19	4	−0.92	0.411	
	June 2020	8.59	±	0.06	8.65	±	0.07	0.06	±	0.14	4	1.06	0.348	
Legionella spp. (*ssrA*)	Aug. 2019	3.41	±	0.29	3.46	±	0.45	0.05	±	0.85	4	0.15	0.886	
	Oct. 2019	4.03	±	0.10	3.72	±	0.03	−0.30	±	0.17	4	−4.87	0.008	**
	May 2020	3.40	±	0.13	3.51	±	0.21	0.11	±	0.39	4	0.78	0.481	
	June 2020	3.25	±	0.08	3.12	±	0.59	−0.13	±	0.95	4	−0.38	0.726	
							Lake B							
total bacteria (16S rRNA genes)	Aug. 2019	8.46	±	0.04	8.72	±	0.07	0.26	±	0.14	4	5.30	0.006	**
	Oct. 2019	8.60	±	0.12	8.61	±	0.11	0.01	±	0.26	4	0.10	0.924	
	May 2020	8.62	±	0.04	8.58	±	0.06	−0.04	±	0.12	4	−0.84	0.450	
	June 2020	8.55	±	0.06	8.66	±	0.05	0.11	±	0.12	4	2.45	0.070	.
Legionella spp. (*ssrA*)	Aug. 2019	3.98	±	0.38	3.97	±	0.13	−0.01	±	0.65	4	−0.05	0.965	
	Oct. 2019	4.10	±	0.05	3.83	±	0.19	−0.27	±	0.32	4	−2.31	0.082	.
	May 2020	3.51	±	0.06	3.43	±	0.09	−0.08	±	0.18	4	−1.28	0.270	
	June 2020	3.52	±	0.06	3.57	±	0.04	0.05	±	0.12	4	1.16	0.311	

[a]Significance codes for *p*: "***" ≤0.001; "**" ≤0.01; "*" ≤0.05; "." ≤0.1

(AIC). The linear model with the best fit (if any) was selected for subsequent hypothesis testing.

RESULTS

Water Temperature and Thermal Stratification. Thermal stratification was observed for six of the eight sampling events for lake A and six of seven events for lake B (Figure 1). Temperature was inversely correlated with depth in lake A during these events and also in April 2020, despite no thermocline during that event (Spearman's ρ ranging from −0.80 to −0.99, $p < 0.05$; see Figure S2 for full results). The thermocline depth varied from 11 to 20 m (median 16 m; Table S2) in lake A and 6 to 19 m (median 10 m) in lake B, and the thermocline in lake A was on average 5 m deeper than that in lake B ($p = 0.01$).

Despite differences in the thermoclines, water temperature at the lake surface did not significantly differ between the two lakes overall (mean difference = 0.1 °C, $p = 0.67$; see Table S5 in the Supporting Information). The peak surface water temperatures were 18.0 °C for lake A (August 2020) and 18.5 °C for lake B (July 2020), and during the non-winter period, lake B was on average 0.9 °C warmer on its surface ($p = 0.03$, paired *t*-test), but only at the surface. Furthermore, during winter (December to March), ice cover was only observed on lake B. The highest temperatures observed at the intakes were 6.1 °C for lake A (June 2020) and 7.0 °C for lake B (September 2019), greater than the annual average temperatures at these depths, 4.1 ± 1.6 °C (mean ± standard deviation for lake A) and 4.6 ± 2.0 °C (lake B). Water temperature did not differ between the water intakes, despite their different depths (mean pairwise difference = −0.4 °C and $p = 0.27$).

Water Quality. Lake DO, pH, conductivity, turbidity, and TOC/DOC versus depth are summarized in Fig. S1 of the Supporting Information, and statistical contrasts between the two lakes are fully described in Table S6. In summary, lake A had on average slightly lower DO than lake B (9.9 ± 1.3 vs 10.2 ± 1.4 mg/L), higher TOC and DOC (3.1 ± 0.2 vs 2.1 ± 0.2 mg/L TOC and 3.0 ± 0.1 vs 2.0 ± 0.1 mg/L DOC), and was slightly

more acidic (pH 7.3 ± 0.2 vs 7.6 ± 0.2) and less conductive (57.3 ± 2.0 vs 94 ± 3 μS/cm). There was no apparent difference in turbidity between the two lakes (0.5 ± 0.2 vs 0.4 ± 2.0 FNU).

In lake A, many of the parameters at least occasionally correlated with lake depth, and the full Spearman's rank tests are summarized in Figure S2. Notably, DO was negatively correlated with lake depth in August 2019 and May and July 2020, while the other parameters were sometimes positively correlated with lake depth: pH in September 2019, conductivity in August 2019 and April 2020, turbidity in August and September 2019, TOC in August and October 2019 and April 2020, and DOC in August and October 2019. Notably, most of the TOC was present in the form of DOC. Seasonal trends were not readily apparent among the various parameters, likely because despite these occasionally significant correlations, the mean differences in water quality between the lake surfaces and drinking water intakes were not large in either lake (Table 1). The sample size may also limit our statistical power to detect such relationships.

Differences Between the Two Drinking Water Sources. Total bacteria (as 16S rRNA gene copies) ranged from 8.27 to 8.79 $\log_{10}[\text{copies/L}]$ in lake A (median 8.55) and 8.43 to 8.89 $\log_{10}[\text{copies/L}]$ in lake B (median 8.61) (Figure 2), and the concentration in lake B was about 0.07 $\log_{10}[\text{copies/L}]$ higher than that in lake A ($p < 0.001$, 95% confidence interval (CI) [0.03, 0.11]).

Legionella spp. (as *ssrA* copies) were consistently detected via qPCR in both lakes (Figure 2), ranging from 2.33 to 4.14 $\log_{10}[\text{copies/L}]$ in lake A (median 3.51 $\log_{10}[\text{copies/L}]$) and from 2.69 to 4.27 $\log_{10}[\text{copies/L}]$ in lake B (median 3.58 $\log_{10}[\text{copies/L}]$). The concentration in lake A was on average 0.2 $\log_{10}[\text{copies/L}]$ less than that in lake B ($p < 0.001$, 95% CI [0.1, 0.3]).

In contrast to the prevalence of *Legionella* spp., *L. pneumophila* was never detected by qPCR (as *mip*) in either lake and only twice by Legiolert (of 90 unique site/depth collection events or 248 samples with replicates). These two culture-positive samples were both in lake B during the August 2019 collection event, at depths of 10 and 20 m and at concentrations of 200 and

110 "most probable number" (MPN) per 100 mL, respectively. DNA recovered from one random positive well per each positive Quanti-Tray, however, exhibited no amplification for *mip* via qPCR. As with *mip*, *Acanthamoeba* spp. (as 18S rRNA genes) were never detected by qPCR.

Differences between Water at the Surface and at Drinking Water Intake. In lake A, total bacteria concentrations at the lake surface and at water intake did not differ during three of the four sample collection events (Table 2); the exception was October 2019, when the concentration at the water intake was about 0.2 $\log_{10}[\text{copies/L}]$ lower than that at the lake surface ($p = 0.031$). In lake B, concentrations on the lake surface and at water intake did not differ in October 2019 or May 2020 (Table 2), but the concentration was about 0.3 $\log_{10}[\text{copies/L}]$ higher at the intake than at the lake surface in August 2019 ($p = 0.006$) and potentially again in June 2020 (0.1 $\log_{10}[\text{copies/L}]$ higher, $p = 0.070$).

Legionella spp. concentrations were about 0.3 $\log_{10}[\text{copies/L}]$ higher at the lake surfaces than at the respective water intakes during October 2019, although this difference was only significant in lake A ($p = 0.008$ vs $p = 0.082$ in lake B; Table 2). In both lakes, the concentrations were not statistically discernible between the surface and water intake depths during the other three sample collection events (August 2019 and May or June 2020).

Explanatory Power of Lake Depth or Thermal Layers on Seasonal Concentrations. Model selection and goodness-of-fit are summarized in Table S7 in the Supporting Information, while hypothesis tests are fully described in Table S8.

Total Bacteria (16S rRNA Genes). August 2019 and June 2020 (Summer). Total bacterial concentrations in lake A were best explained with the thermal stratification layer in August 2019 (adj. $R^2 = 0.19$ and $p = 0.028$). The hypolimnion concentration, however, was only about 0.1 $\log_{10}[\text{copies/L}]$ lower than the upper stratum concentration, and this difference was not significant ($p = 0.064$). Neither lake depth nor thermal stratification explained lake A total bacteria in June 2020. June 2020 was early summer, however, while August 2019 was later in summer, which was reflected in the lake temperature profiles (Figure 1).

In contrast to lake A, the lake B total bacterial concentration was best explained by lake depth in August 2019 (adj. $R^2 = 0.66$ and $p = 0.001$). Lake strata also fit these data—albeit with higher AIC—so the difference in model selection (vs lake A) may be partly due to the smaller sample size in lake B. Nonetheless, unlike lake A, total bacteria in lake B increased by about 0.09 $\log_{10}[\text{copies/L}]$ per 10 m depth interval. Like lake A, however, none of the four models explained total bacteria in June 2020.

October 2019 (Autumn). Lake A concentrations were best explained by thermal stratification (adj. $R^2 = 0.67$ and $p < 0.001$), and the hypolimnion concentration was about 0.2 $\log_{10}[\text{copies/L}]$ lower than the upper stratum concentration ($p = 0.001$). In contrast, none of the models explained total bacteria in lake B.

May 2020 (Spring). Neither lake A nor lake B total bacteria concentrations were well-explained by lake depth or thermal stratification in May 2020, which was consistent with both lakes being thermally destratified during this period (Figure 1).

Legionella spp. (ssrA). August 2019 and June 2020 (Summer). In lake A, *Legionella* spp. concentrations in August 2019 were best explained by thermal stratification (adj. $R^2 = 0.29$ and $p = 0.007$), but the hypolimnion concentration overall was actually about 0.4 $\log_{10}[\text{copies/L}]$ higher than the upper stratum concentration ($p = 0.037$). This result is in contrast to the lake A

total bacteria concentration (lower in the hypolimnion) and in contrast to lake A surface concentration versus its water intake (no difference). Both the complex models (lake depth plus the thermal layer, with or without an effect of depth within each layer) were also of significant albeit poorer fit. Thus, the distribution of *Legionella* spp. within and between thermal layers may be more complex than the simpler model suggests; selection of the simpler model may be due to the limited sample size and statistical power.

Interestingly, although concentrations in lake B were best described using a linear model incorporating both lake depth and thermal stratification in August 2019, subsequent hypothesis testing—despite a mean concentration about 0.5 $\log_{10}[\text{copies/L}]$ higher in the upper stratum relative to the hypolimnion—was not statistically conclusive ($p = 0.389$ and CI [−1.4, 2.4]; Table S8).

As for total bacteria, however, *Legionella* spp. concentrations during June 2020 were not well explained by any of the four models in either lake (adj. $R^2 < 0.3$ and $p > 0.1$; Tables S7 and S8).

October 2019 (Autumn). *Legionella* spp. concentrations in lake A were best explained by the most complex model: an effect of lake depth within each thermal layer (adj. $R^2 = 0.46$ and $p = 0.003$). In the upper thermal layer, concentration decreased with lake depth (about −0.5 $\log_{10}[\text{copies/L}]$ per 10 m; $p = 0.016$), while in the hypolimnion, there was a less convincing association with lake depth (about −0.1 $\log_{10}[\text{copies/L}]$ per 10 m; $p = 0.057$).

In lake B, the most complex model was not considered (there was only one depth within the hypolimnion), and of the three models considered, concentrations were best explained by lake depth alone (adj. $R^2 = 0.35$, $p = 0.024$). *Legionella* spp. concentrations decreased by about 0.1 $\log_{10}[\text{copies/L}]$ per 10 m ($p = 0.024$).

May 2020 (Spring). Similar to the total bacteria, *Legionella* spp. concentrations in the two lakes were not well explained by any of the models (adj. $R^2 < 0.3$ and $p > 0.1$; Tables S7 and S8).

■ DISCUSSION

The practice of utilizing the catchment as a hygienic barrier in Norway may have empirical support with regard to certain fecal indicator organisms such as total coliforms and *Escherichia coli*.[2,4] Although both the origin and pathogenicity of fecal organisms differ from those of naturally occurring legionellae,[53] there were ecological, epidemiological, and empirical reasons to suspect that a barrier-like effect may extend to legionellae by way of lake depth or seasonal thermal stratification.[35,39,40,54]

In this study, however, total *Legionella* concentrations at the drinking water intake were typically similar to those at the lake surface, even when the lake was thermally stratified. Concentrations differed between these two depths in autumn, but the concentration observed in the hypolimnion was only 0.3 $\log_{10}[\text{copies/L}]$ or less than that at the lake surface. Similar trends were observed for total bacteria concentrations. More broadly, although *Legionella* concentrations were seasonally associated with the lake depth and thermal layer—perhaps mirroring seasonality of *Legionella* in other contexts[36,54,55]—it was unclear whether placement of the drinking water intake at any depth within the hypolimnion provides a substantial log-reduction benefit against environmental *Legionella* spp.—especially pathogenic *L. pneumophila*, which was never observed by PCR or cultivation in either lake. Other than the differences in lake depth between the two lakes, another important factor

not fully addressed by this investigation was the proximity of the water intake to the lake bottom. The intake for lake A is at least 10 m above the lake sediment, while in lake B, it is within 2 m. Proximity to either the lake surface or lake bottom plays an important role in bacterial community composition across the water column,[26,56−58] and both *Legionella* and their amoeba hosts have been observed in lake sediments.[20,59]

The use of multiple secondary or reserve water supplies is common in Norway,[60] and as municipalities supplement their drinking water supply with new sources—either in response to increasing consumer demand or in anticipation of future water scarcity (e.g., due to climate change)—the blending of different waters of variable quality could have implications for *Legionella* management in buildings. For example, the intermittent switching of the municipal water source, either partially or completely, could complicate routine *Legionella* monitoring or stymie infection source tracking. Extreme actions such as changing the entire municipal water source have been linked to unforeseen adverse consequences in the distribution network, such as the spike in lead levels in the Flint, Michigan water supply and concurrent increase in Legionnaires' disease incidence in the city.[61,62]

In the present study, there were minor differences in some water quality parameters between the two drinking water sources: DO, pH, conductivity, TOC, and DOC. We did not directly assess the relationships of these parameters to the taxa of interest, but concentrations of total bacteria and *Legionella* spp. were slightly higher in lake B. We also did not assess whether the microbiological differences between water sources carry forward into the municipal distribution network after water treatment. Importantly, although *L. pneumophila* was never detected, we did not further differentiate the non-*L. pneumophila* species. Important differences may still exist between the two water sources with regard to *Legionella* diversity and more than 20 non-*pneumophila* pathogenic *Legionella*.[11,14,18,54,63]

Of course, physical and chemical treatment of raw water may significantly reduce the diversity and abundance of *Legionella*, such that the legionellae composition in tap water is highly distinct from the source waters.[64] Because water treatment for this Norwegian municipality—and many others in Norway—does not utilize physical–chemical treatment processes like coagulation, sedimentation, and filtration,[60,65] concerns remain about the efficacy of disinfection alone to manage potential intrusion of pathogenic *Legionella* (or their hosts)[66,67] into the distribution network via surface water sources. For example, various common freshwater plankton have been associated with survival of bacteria through UV and chlorine disinfection.[68−70] *Legionella* can also survive and remain infectious after UV exposure,[71] and despite the relative efficacy of free chlorine against planktonic *Legionella*, this efficacy relies on sustained contact with a sufficient residual concentration,[72−74] which is not commonly practiced in Norway.

Compared to the *Legionella* spp. (as *ssrA* copies) concentrations we have previously observed in the municipal tap water (range < 2.6 to 3.4 $\log_{10}$[copies/L], median 3.4),[40] the concentrations in the source waters were similar (medians of 3.5 and 3.6 $\log_{10}$[copies/L] in lakes A and B, respectively). Total bacteria in the municipal tap water ranged from 6.4 to 8.5 $\log_{10}$[copies/L], which were about one order of magnitude lower than those observed in the source waters in this study (medians of about 8.6 $\log_{10}$[copies/L] in both lakes). Thus, while treatment appears to achieve some log reduction of these DNA targets, concentrations at the tap reflect a total log

reduction that is less than what is prescribed in Norway—typically at least 3.0 to 6.0 log removal of bacteria depending on utility size and source water quality.[2] Certainly, some regrowth of bacteria in the distribution network and building plumbing systems[75,76] could contribute to this apparent log reduction deficit.

Finally, although Norway is not a member of the European Union (EU), it adopts and maintains parity with many aspects of the EU regulatory framework via its membership in the European Economic Area (EEA). This includes the recently revised EU drinking water directive that prescribes more *Legionella* monitoring in potable water systems.[77−79] Thus, analytical tools like the Legiolert Quanti-Tray system and other culture-based methods (e.g., ISO 11731) may see increased use for the monitoring of *L. pneumophila* in water supplies sources.[47,48,80,81] In this study, the two positive *L. pneumophila* Quanti-Trays appeared to be false positives as we were unable to detect *L. pneumophila* via qPCR in DNA extracted from positive wells. The *mip* primers used in the qPCR reactions have been validated for at least 145 environmental and clinical isolates of *L. pneumophila*,[45,82] and validated in our laboratory using *L. pneumophila* genomic DNA (ATCC 33152D).[40] Although the manufacturer of the Legiolert method provides separate protocols for potable and non-potable water—primarily to minimize false positives via interfering microorganisms—this investigation highlights the need for subsequent confirmation via more selective methods (e.g., DNA-based methods). Considering the relatively restrained approach to water treatment in Norway and some other European countries compared to, for example, the United States, this also raises questions about the delineation of potable versus non-potable for the purposes of the Legiolert method because potable water without residual disinfectant may have higher bacterial biomass and diversity than potable water with residual disinfectant.[41]

■ CONCLUSIONS

In two Norwegian lakes, planktonic concentrations of bacteria and especially environmental *Legionella* may correspond seasonally to lake depth or the development of thermal stratification layers in the late summer or autumn. The associations with lake depth, however, were relatively weak, with concentrations at the water intake differing by less than 0.5 log relative to those at the lake surfaces. Although placing the drinking water treatment plant intakes deeper within the lake hypolimnions may provide a hygienic barrier against pathogens of fecal origin, this barrier-like effect may not extend to naturally occurring legionellae. Furthermore, the utilization of multiple water sources—servicing the same municipal distribution network—may nonetheless result in regional microbiological differences in tap water.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.1c00454.

> Details about statistical treatment and hypothesis testing, water quality versus depth in Norwegian Lakes A and B during the monitoring period, pairwise Spearman's rank correlation tests among the water quality parameters and lake depth in Norwegian Lake A, physical characteristics of lakes A and B, depth profile and sample summaries, qPCR primers/probes and protocols, template sequences

for synthetic qPCR standards, summaries of real-time qPCR standard curves, water quality in lakes A and B, linear model selection prior to hypothesis testing of genetic marker concentrations, and hypothesis testing of genetic target concentrations via linear regression (PDF)

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Notes

The authors declare no competing financial interest.

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