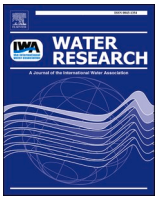
Water Research 253 (2024) 121109

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres



Domestic hot-water boilers harbour active thermophilic bacterial communities distinctly different from those in the cold-water supply

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ARTICLE INFO

Keywords:
Drinking water
Hot-water boilers
Microbiome composition
Thermophilic growth
Flow cytometry
Assimilable organic carbon

ABSTRACT

Running cold and hot water in buildings is a widely established commodity. However, interests regarding hygiene and microbiological aspects had so far been focussed on cold water. Little attention has been given to the microbiology of domestic hot-water installations (DHWIs), except for aspects of pathogenic *Legionella*. Worldwide, regulations consider hot (or warm) water as 'heated drinking water' that must comply (cold) drinking water (DW) standards. However, the few reports that exist indicate presence and growth of microbial flora in DHWIs, even when supplied with water with disinfectant residual. Using flow cytometric (FCM) total cell counting (TCC), FCM-fingerprinting, and 16S rRNA-gene-based metagenomic analysis, the characteristics and composition of bacterial communities in cold drinking water (DW) and hot water from associated boilers (operating at 50 – 60 °C) was studied in 14 selected inhouse DW installations located in Switzerland and Austria. A sampling strategy was applied that ensured access to the bulk water phase of both, supplied cold DW and produced hot boiler water. Generally, 1.3- to 8-fold enhanced TCCs were recorded in hot water compared to those in the supplied cold DW. FCM-fingerprints of cold and corresponding hot water from individual buildings indicated different composition of cold- and hot-water microbial floras. Also, hot waters from each of the boilers sampled had its own individual FCM-fingerprint. 16S rRNA-gene-based metagenomic analysis confirmed the marked differences in composition of microbiomes. E.g., in three neighbouring houses supplied from the same public network pipe each hot-water boiler contained its own thermophilic bacterial flora. Generally, bacterial diversity in cold DW was broad, that in hot water was restricted, with mostly thermophilic strains from the families *Hydrogenophilaceae, Nitrosomonadaceae* and *Thermaceae* dominating. Batch growth assays, consisting of cold DW heated up to 50 – 60 °C and inoculated with hot water, resulted in immediate cell growth with doubling times between 5 and 10 h. When cold DW was used as an inoculum no significant growth was observed. Even boilers supplied with UVC-treated cold DW contained an actively growing microbial flora, suggesting such hot-water systems as autonomously operating, thermophilic bioreactors. The generation of assimilable organic carbon from dissolved organic carbon due to heating appears to be the driver for growth of thermophilic microbial communities. Our report suggests that a man-made microbial ecosystem, very close to us all and of potential hygienic importance, may have been overlooked so far. Despite consumers having been exposed to microbial hot-water flora for a long time, with no major pathogens so far been associated specifically with hot-water usage (except for *Legionella*), the role of harmless thermophiles and their interaction with potential human pathogens able to grow at elevated temperatures in DHWIs remains to be investigated.

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https://doi.org/10.1016/j.watres.2024.121109
Received 28 October 2023; Received in revised form 29 December 2023; Accepted 4 January 2024
Available online 6 January 2024

Abbreviations

AOC	assimilable organic carbon
D(H)WI	domestic (hot-)water installation
DOC	dissolved organic carbon
DW	drinking water
FCM	flow cytometry / flow-cytometric
TCC	total cell count (cells mL^{-1})
TCC_c	total cell count in cold water (cells mL^{-1})
TCC_h	total cell count in hot water (cells mL^{-1})
$TCC_{(h/c)}$	ratio TCC_h/TCC_c
t_d	doubling time (h)

1. Introduction

For more than a century, running cold and hot water in buildings has been a widely established commodity. However, interests regarding hygiene and microbiological safety had always been focussed on cold water (Berry et al., 2006). Except for potentially pathogenic *Legionella* (Flemming et al., 2014; Van der Kooij, 2014), little attention has been given to the microbiology of domestic hot-water installations (DHWIs). As far as we are aware, this also applies for drinking water (DW) regulations worldwide. According to Swiss Regulations (TBDV 2021), 'warm water' is defined as 'heated DW'. EU regulations (EU, 2024) only refer to 'water intended for human consumption' in 'domestic distribution systems', which includes any installation between the point where DW enters the building and the consumer's tap. In EU regulations, the US report (NASEM 2019) and WHO guidelines (WHO, 2017), warm or hot water is not mentioned, except for specific microbiological aspects of hot water (e.g., baths), which focus on the presence of *Legionella pneumophila*. Hence, if mentioned at all, hot water is defined as 'heated DW', and accordingly, must comply established (cold) DW standards.

First reports on the occurrence of microbial cells in DHWI date back to the 1970s when thermophilic Gram-negative *Thermus aquaticus*-like strains were isolated from water heaters and hot tap-water (Brock and Boylen, 1973; Pask-Hughes and Williams, 1975; Stramer and Starzyk, 1981). Later, reports documented high numbers of microbial cells in hot-water systems in private and public buildings (Baron et al., 2014; Ji et al., 2017; Zacheus and Martikainen, 1995), often exceeding levels found in the respective cold water (Bagh et al., 2004; Bagh et al., 1999; Ley et al., 2020). Recently, frequent presence of *Thermus scotoductus* strains was observed in domestic water heaters throughout the US (Wilpiszeski et al., 2019).

With exceptions (Baron et al., 2014; Props et al., 2016), systematic examinations of hot-water microbiomes in DHWIs are largely lacking. When starting this work, we were aware of one report only that compared a hot-water microbiome to that of the supplied cold water (Henne et al., 2013). These authors found highly different bacterial communities in cold and hot water in a laboratory building. Both contained similar concentrations of bacterial cells, but compared to the cold-water community, that in hot water exhibited a much lower diversity with phylotypes mostly related to bacteria found in high-temperature habitats. Furthermore, a high RNA content of the hot-water community suggested fast growth. The high seasonal variability of the cold-water microbiome contrasted with the high stability of that in hot water. Several of these observations were confirmed recently (Ley et al., 2020; Meyer et al., 2023).

Despite consumers having been exposed to hot-water flora for a long time, except for *Legionella*, no major pathogens have so far been associated specifically with hot-water usage and consumption. However, the lack of information, hygienic regulations, and the fact that cold DW and hot water produced thereof might harbour completely different microbiological microbiomes asks for closer inspection and careful microbiological investigation of DHWIs. Thus, the goal of our study was to investigate and characterize the bacterial communities in a variety of DHWIs, based on total cell counts (TCC), flow cytometric fingerprinting, growth potential and community composition. Comparing supplied cold DW with hot water produced thereof, we wanted to investigate whether boilers are unique ecosystems with unique thermophilic microbiomes, and propose, if possible, a reason for observed thermophilic growth.

2. Materials and methods

2.1. Study design

Two sampling campaigns were performed to investigate the presence and characteristics of hot-water microbiomes in DHWIs under real conditions. The goal of a first screening campaign was to gather information on whether the observation reported by Henne and colleagues (Henne et al., 2013) is widespread or restricted to individual buildings. We used flow cytometry (FCM) for rapidly screening microbial abundance and FCM-fingerprints in cold- and hot-water samples collected in DHWIs. To obtain comparable results, automated portable flow cytometers (BactoSense equipped with a TCC cartridge) that produce standardized measurements were used. Furthermore, to investigate whether the microbial hot-water flora present in the bulk water phase of boilers is indeed active and able to grow fast at boiler temperatures, as suggested earlier (Henne et al., 2013), *ex-situ* batch growth assays were performed to allow controlled experimentation.

Preliminary tests in a private building confirmed remarkable differences between cold DW and hot water produced thereof (Supplementary Materials, Fig. S1): Whereas cellular concentrations and fingerprints of cold water supplied were very similar, hot water from the boiler contained roughly ten-times more cells and had a very different FCM-fingerprint. This suggested microbial growth in the hot-water installation and presence of microbial flora distinctly different from that in the supplied cold DW. After a first sampling campaign confirmed that the phenomenon is indeed widespread, a second campaign followed to document the findings in public and private buildings located in different municipalities. Locations were specially selected to cover a wide range of boiler sizes and ages. During this second sampling campaign cold- and hot-water samples for 16S rRNA-gene-based metagenomic analysis were collected, along with samples for FCM-analyses.

2.2. Selected locations, and sampling campaigns

Locations chosen for sampling were domestic DW installations comprising boilers in communities in the regions of Zurich, Central Switzerland, as well as Lower Austria, and Vienna. Preliminary samples were collected in autumn 2018 (e.g., Supplementary Materials, Fig. S1). During the first sampling campaign including more than 20 locations (spring to autumn 2020) differences observed in TCCs and FCM-fingerprints in cold and hot water were confirmed. From these, 14 locations were selected for closer investigation, plus two controls. Selection criteria included easy and constant accessibility for the sampling team, covering a wide range of boilers of different ages, sizes, and surface to volume (S/V) ratios (Table 1), but also the opportunity to compare hot water from boilers in different buildings supplied with the same cold water (locations 1–4). During the second campaign (April 18 until May 24, 2022), samples for FCM and 16S rRNA-gene-based community analyses were collected (Tables 1, 2). The first control (location 18) had a flow-through heater installed (without boiler). The second control (location 15) was the communal lake water treatment plant supplying DW for locations 1–4. Here, the sample was collected at the production plant right after UVC treatment, directly before distribution; therefore, only a cold DW sample is available. This facility introduced UVC-treatment in December 2021; before, rapid sand filtration was the final treatment.

Table 1
List of locations sampled and characteristics of boilers.

Location, Sample #	Community	Boiler V (L)	S/V (cm^{-1})	Boiler age (years)
1	CH-8706 Meilen	500	0.08	10
2	CH-8706 Meilen	500	0.08	5
3	CH-8706 Meilen	600	0.08	>20
4	CH-8706 Meilen	300	0.09	17
5	CH-8700 Küsnacht	1000	0.06	>10
7	CH-5200 Brugg	200	0.10	22
8	CH-5200 Brugg	400	0.09	3
9	CH-8032 Zürich	1000	0.06	17
11	CH-6463 Bürglen*	800	0.07	>30
12	CH-8045 Zürich	15	0.60	2
13	CH-8032 Zürich	200	0.10	>10
15	CH-8706 Meilen	Control	–	
16	A-2135 Neudorf	200	0.10	>29
17	A-2135 Neudorf	80	0.14	14
18	A-3011 Irenental	Control	–	
19	A-1090 Vienna	300	0.09	>19

Detailed information on locations and boilers sampled for FCM and 16S rRNA-gene-based community analyses during the second sampling campaign (April 18 until May 24, 2022). Controls: # 15 represents UVC-treated DW leaving the plant and supplying locations 1–4 (cold-water sample only) and at # 18 samples were collected before and after a flow-through heater installation. Numbers 6, 10 and 14 were assigned to blanks during community analysis. The inner surface of all boilers consisted of vitreous enamel, except at location 16 (copper). V = volume; S/V = surface/volume ratio; * heating and water installation completely replaced in autumn 2022.

2.3. Flow cytometric TCCs and fingerprints

Bacterial cell abundance and FCM-fingerprints (i.e., the pattern of data points observed in 2-dimensional FCM-plots) were quantified using a total cell count (TCC) method. It was preferred to intact cell counting ('live-dead') for several reasons. First, a standardized, officially accepted protocol of a TCC method is available (see e.g., the Swiss method: https://www.svgw.ch/wasser/methodenplattform/methodenkatalog/), whereas no officially standardized ICC method has been reported yet. Second, TCC detects all cells and matches the data obtained from 16S rDNA-gene amplicon sequencing, which also catches all cells, alive and dead. Third, TCC was preferred because most of the selected locations were supplied with UVC-treated DW, and the ICC method is well-known to be unable to assess 'viability' of UVC-treated cells. For all flow cytometric measurements BactoSense, a transportable, fully automated online flow cytometer (bNovate Technologies SA, CH-1024 Ecublens, Switzerland) equipped with a cartridge for TCC determination (with SYBR Green I) was used in the off-line mode. This instrument uses the Swiss standard TCC-staining protocol and contains a blue 488 nm laser diode and three detectors for measuring green fluorescence signals at 535/43 nm (FL1), red fluorescence signals above 750 nm LP (FL2), and 488/10 nm side scatter signals (SSC). It produces very reproducible results such that TCCs and FCM-fingerprints determined for the same water with different instruments can be compared (for 3 different instruments, the coefficients of variation (CV) for triplicate determinations of TCC for a bottled still water and a groundwater were 1.1 % and 2.9 %, respectively, for HNA% the CVs were 1.7 and 1.8 %, respectively). If not mentioned specifically, the instrument's default gates were used for TCC determination. The usually used differentiation between low nucleic acid (LNA) and high nucleic acid (HNA) content cell clusters was not reported because in many of the samples cell clusters could not be allocated clearly to one of these clusters (see Fig. 1).

2.4. Sampling strategy and reproducibility of FCM data

Because DWIs are often of hidden complexity, we used a sampling strategy that allows access to the bulk water phase of both, supplied cold DW and produced hot boiler water, avoiding samples being affected by stagnation. Water samples were always collected from taps directly; the only exception was location 1 where, for comparison, the incoming cold DW was also sampled directly after entering the building. Samples were collected in sterile 50 mL Falcon tubes, which were first rinsed 5-times with running water from the tap before the sample was drawn. If not directly analysed, tubes were immediately cooled down to 4 °C, kept at this temperature and analysed within < 24 h. As a rule, the standard deviation of TCCs determined for identical samples of both cold and hot water was < 6 % (Supplementary Materials, Table S1, Fig. S2). Repeated sampling during a day or over longer periods of time demonstrated considerable stability of TCC in both, cold DW supplied and hot water from the associated boiler (Supplementary Materials, Fig. S9).

To minimize effects of stagnant water in pipes on TCCs and FCM-fingerprints, water temperature was taken as an indication of complete flushing, i.e., taps sampled were flushed until water temperature remained constant for more than 15 s. The maximum flow velocity was always < 0.8 m sec^{-1} to avoid detachment of biofilm particles during flushing (El-Chakhtoura et al., 2018). Two examples support this statement. First, in three neighbouring buildings connected to the same DW mains (but different link-up), TCCs and FCM-fingerprints of cold DW sampled from the taps were essentially identical (Fig. 2). Second, at location 1 cold water was sampled right after entering the building (approx. 2 m from the communal DW supply mains) and at a tap on the 3rd floor (compare Supplementary Materials, Fig. S10). Also here, TCC concentrations and fingerprints were virtually identical after entering the building and at the tap (Supplementary Materials, Fig. S2). This suggests that the influence of biofilms and regrowth in stagnant water was avoided with this sampling strategy, and that TCCs and FCM-fingerprints of water samples collected from household taps corresponded to those found in the communal network, and from the boiler, respectively. This is also supported by the recent finding that even highly dynamic hydraulic conditions had no detectable influence on FCM-determined suspended microbial cell concentrations (Prest et al., 2021).

2.5. Bray-Curtis dissimilarity analysis of FCM fingerprints

The flow cytometric data were acquired as Flow Cytometry Standard (FCS) files. Data were preprocessed employing the instrument's default gates to eliminate noise and debris (see 2.2. and Fig. 2). Data from gated events were normalized using Fast Fourier Transform (FFT) to ensure that the subsequent fingerprinting process would not be influenced by variations in TCCs. Subsequently, a three-dimensional FCM-fingerprint was computed for each sample using a three-dimensional Kernel Density Estimate (KDE) applied to the fluorescence (FL1, FL2) and side-scatter (SSC) distributions, as described earlier (De Roy et al., 2012; Favere et al., 2020).

Bray-Curtis (BC) dissimilarity metrics was used to assess dissimilarities between these fingerprints (Bray and Curtis, 1957; Buysschaert et al., 2019; Favere et al., 2020). Analysis of similarity (ANOSIM) was employed to quantify dissimilarities between bacterial communities in hot- and cold-water samples, leveraging data from the BC dissimilarity matrix (Supplementary Materials, Table S2). Additionally, BC pairwise dissimilarities were examined between samples from the origin, as well as inter-replicate BC dissimilarity. The BC dissimilarity matrix was then subjected to two-dimensional Principal Component Analysis (PCA) to reduce data dimensionality, making it easier to interpret data when specific population characteristics are not well-defined.

Furthermore, to detect the presence of a hot-water bacterial community within unknown samples, PCA was employed to project the 3D-FCM fingerprint data, and then a Support Vector Machine (SVM) classifier was trained on this dataset. Due to limited availability of training data, we assessed the classifier's performance using leave-one-out cross-validation.

2.6. Growth experiments

The ability of microbial hot-water and cold-DW flora to grow in DW as a growth medium at room or boiler temperature was tested in *ex-situ* batch assays. If not stated otherwise, water from location 1 was used. Here, the TCC in hot water from the boiler was always approximately 3-times higher than in the supplied cold DW (Table 2; Supplementary Materials, Fig. S9); therefore, growth was expected to occur in cold DW when shifted to boiler temperature (~55 °C), either on its own, or after inoculation with hot-water flora.

First batch growth assays were performed in a stainless-steel container (3 L). No growth was observed in cold DW heated up to 55 °C and incubated at this temperature in the dark for 24 h. However, when assays were supplemented with an aliquot of hot water fast batch growth with a more than two-fold increase of TCC was observed (Supplementary Materials, Fig. S4).

To reduce the masking effect of 'non-growing' cold-water flora and to extend the potential batch growth range from inoculation to stationary phase, assays were also performed with 0.22 μm-filtered cold water, as the growth medium, removing ~90 % of the cells. Before use, filters were rinsed with 20 mL of cold DW to avoid transfer of AOC from filters into the assay. Typically, Falcon tubes (60 mL) were used as reactors. After extensive rinsing with 0.22 μm-filtered cold DW, they were aliquoted with 40–50 mL of filtered cold DW and amended with either 10–20 mL of hot water, or 10–20 mL of cold DW, respectively. Inoculated tubes were immediately placed in a water bath kept in the dark at indicated temperatures (50 – 60 °C, or 20 – 30 °C, respectively). To follow growth, samples of 5 mL were collected as a function of time and TCC was determined immediately. No significant release of growth-supporting nutrients from Falcon tubes was expected that might have influenced the results obtained, because Falcon tubes have been used extensively for the determination of bacterial growth via ^{3}H-thymidine incorporation where they were shown to have no influence on bacterial growth rates (Karner and Herndl, 1992). This is confirmed by results obtained from batch assays performed in stainless-steel containers (Supplementary Materials, Fig. S4) where growth of hot-water flora proceeded with similar doubling times and to a similar extent as those made in Falcon tubes. Furthermore, the extent of '*ex-situ*' batch growth observed from inoculation to the stationary phase was always in the range of 1.5 to 2-fold (see Supplementary Materials, Figs. S4 and S6). This corresponds well with the observed $TCC_{(h/c)}$-ratio listed in Table 2 for cold- and hot-water samples collected at locations 1–4, as well as the long-term boiler $TCC_{(h/c)}$-ratio of 3.8 documented at location 1 (Supplementary Materials, Fig. S9).

2.7. Sample collection for 16S rRNA-gene-based bacterial community analyses

Samples for microbial community analysis were collected in sterile 1 L plastic bottles commercially available for DW-sampling (containing sodium thiosulfate; Gosselin™, Article Nr. HLC25001), or autoclaved 4.2-L Nalgene Heavy-Duty sampling bottles (Nalgene Europe, Hereford, UK). Alongside, a sample was collected for TCC and FCM-fingerprint analysis. The sampling strategy described above was applied and containers were first thoroughly rinsed 5-times with the respective water before a sample was collected. On site, containers with hot-water samples were immediately cooled down to cold-water temperature using running cold water (with occasional shaking this took <10 min). For transport, samples were kept at a temperature <4 °C in cold boxes with cold packs. Samples were processed within <18 h after collection.

Table 2
Information and results from hot- and cold- water samples collected at selected locations between March and June 2022.

Community	Location #	Sample code	Date, time sampled	°C	TCC (cells/mL)*	$TCC_{(h/c)}$	Raw water origin and treatment
CH-8706	1	1h	2022/05/04, 12:00	56.7	407'500	3.11	Lake & spring water > O_3 > RSF > GAC > UVC > DS
		1c	2022/05/04, 12:00	11.2	131'011		
CH-8706	2	2h	2022/05/04, 11:00	57.8	322'688	3.04	Lake & spring water > O_3 > RSF > GAC > UVC > DS
		2c**	2022/05/04, 11:00	11.8	105'977		
CH-8706	3	3h	2022/05/04, 10:15	59.6	227'155	2.05	Lake & spring water > O_3 > RSF > GAC > UVC > DS
		3c	2022/05/04, 10:15	12.6	110'600		
CH-8706	4	4h	2022/05/04, 08:00	51.1	359'544	3.31	Lake & spring water > O_3 > RSF > GAC > UVC > DS
		4c	2022/05/04, 08.00	14.3	108'544		
CH-8700	5	5h**	2022/05/06, 07:00	54.2	186'388	1.72	90% lake water> O_3 > GAC > DS
		5c	2022/05/06, 07:00	11.5	108'644		+ 10% spring water >UVC > DS
CH-5200	7	7h**	2022/05/26, n. r.	64.3	36'966	1.24	92% groundwater > UVC > DS
		7c	2022/05/26, n. r.	17.2	29'755		+ 8% spring water > none > DS
CH-5200	8	8h	2022/05/26, n. r.	50.1	330'544	8.74	92% groundwater > UVC > DS
		8c	2022/05/26, n. r.	16.6	37'822		+ 8% spring water > none > DS
CH-8032	9	9h	2022/05/06, 12:00	61.3	153'477	1.45	Ground- & lake water > O_3 > GAC > SSF > DS
		9c	2022/05/06, 12:00	15.5	105'488		
CH-6463	11	11h	2022/05/13, 08:00	67.5	15'188	1.23	Groundwater > O_3 > GAC > DS
		11c**	2022/05/13, 08:00	10.7	12'344		
CH-8045	12	12h	2022/05/13, 11:30	55.9	169'433	1.52	Ground- & lake water > O_3 > GAC > SSF > DS
		12c	2022/05/13, 09:45	12.4	111'611		
CH-8032	13	13h	2022/05/13, 07:40	55.1	368'466	3.60	Ground- & lake water > O_3 > GAC > SSF > DS
		13c	2022/05/13, 07:30	10.7	80'044		
CH-8706	15	15c	2022/05/25, 14:00	10.0	215'980	-	Lake & spring water > O_3 > GAC > UVC> DS
A-2135	16	16h	2022/04/18, 12:45	52.7	21'111	1.56	Groundwater > part AC, part RO > UVC > DS
		16c	2022/04/18, 12:45	11.0	13'500		
A-2135	17	17h	2022/04/18, 13:30	55.5	15'755	1.11	Groundwater > part AC, part RO > UVC > DS
		17c	2022/04/18, 13:30	12.5	14'211		
A-3011	18	18h	2022/07/21, 07:10	48.5	48'922	0.93	Spring water > UVC > DS
		18c	2022/07/21, 07:10	14.3	52'844		
A-1000	19	19h	2022/07/21, 11:40	60.9	119'666	8.60	Spring water > Cl_2 > DS
		19c	2022/07/21, 11:40	10.8	13'911		

TCCs were determined with Bactosense default gate (no LNA/HNA separation). Samples are identical to those shown in Fig. 1 and Supplementary Materials, Fig. S3. # 6, 10, and 14 were codes used for blanks for filtration for community analysis; h = hot; c = cold; n. r. = Not reported; *Single measurements, variation of TCC with BactoSense typically < ± 5 %. ** = Amplification of DNA not successful. Cl_2 = Chlorination; DS = Distribution system; (G)AC = (Granular) activated carbon filter; O_3 = ozonation; RO = Reverse osmosis; RSF = Rapid sand filtration; SSF = Slow sand filtration; UVC = UVC treatment.

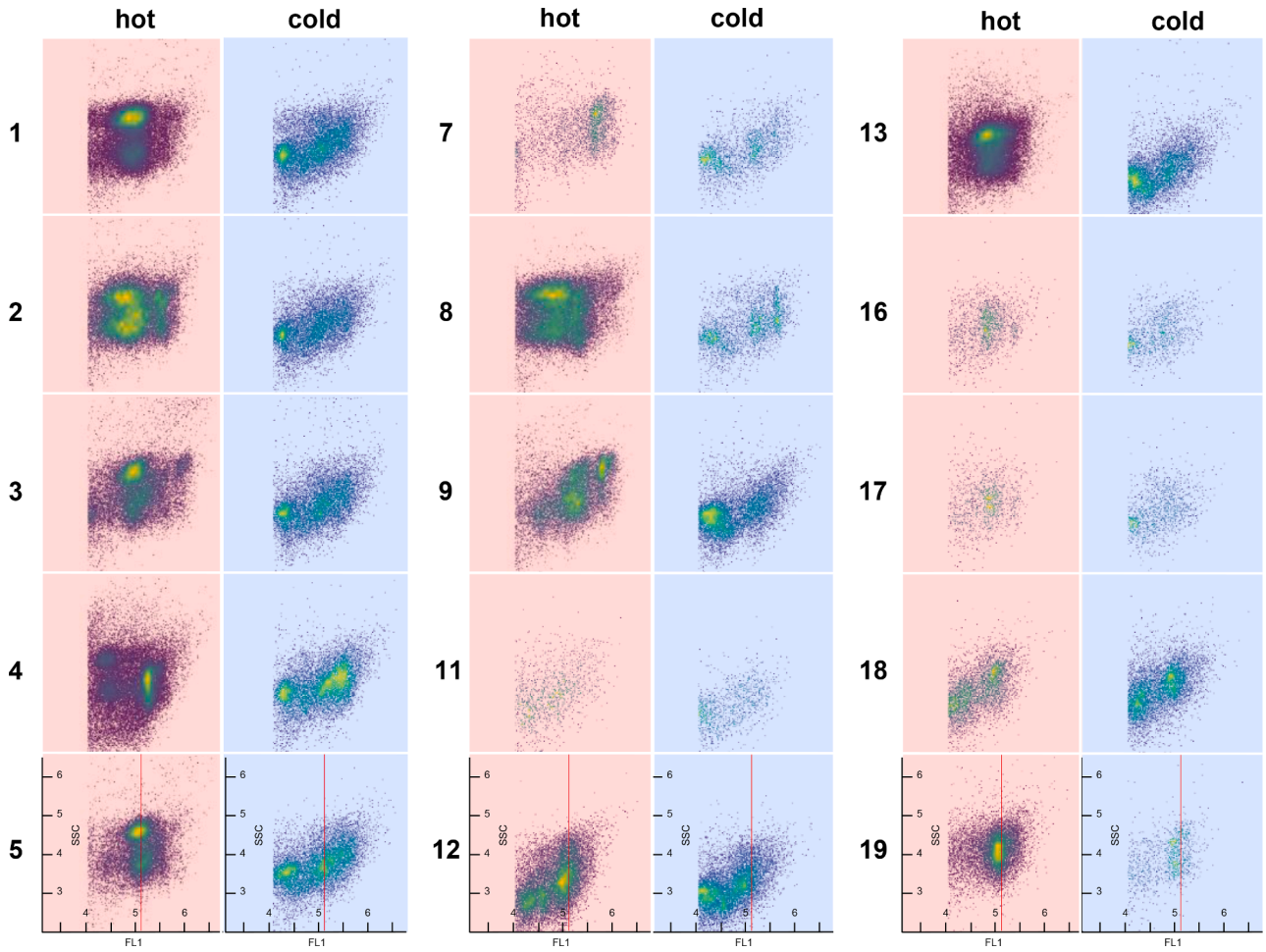

Fig. 1. FCM-fingerprints (FL1-SSC plots) of hot- and cold-water samples collected from all selected locations (for detailed information see Table 1 and 2). Note: Locations missing here were either used as blanks (#6, 10, 14), or consisted of a cold drinking water sample only collected from the treatment plant outlet that supplies water to locations 1–4 (#15). #18 represents samples collected before and after a flow-through heater (used as a control). Scaling and default separation line usually used for determination of LNA and HNA cell clusters are indicated in bottom plots.

For collecting cells for molecular analyses, 1 - 4 litres of sample, depending on its cell concentration, were filtered through a 0.22 µm pore size polycarbonate membrane filter (Isopore™, 47 mm diameter, Millipore, Bedford, USA). The number of cells collected on a filter was determined from the volume of filtrate and the respective TCC. Immediately after filtration, filters with biomass were either frozen and stored at −80 °C (Austrian samples) or stored at −20 °C in sterile Falcon tubes (Zürich samples), transported to Vienna in a cool box at −20 °C and then stored at −80 °C until nucleic acid extraction.

2.8. DNA extraction and PCR

Nucleic acid extraction was performed as described by Griffiths and colleagues (Griffiths et al., 2000) , with a DNA precipitation step using isopropanol instead of polyethylene glycol (Reischer et al., 2008). Recovered DNA was redissolved in 50 µl of sterile bi-distilled water and stored at −80 °C until further analysis. All extracted sample DNAs were checked for amplifiable bacterial DNA and PCR inhibition by applying a general 16S rRNA gene PCR assay (Winter et al., 2007). 16S rRNA gene amplicon sequencing of DNA samples was performed by ARES Genetics, Vienna, Austria.

2.8.1. Sample processing

After assessment of DNA quality, genomic DNA samples were processed with a 2-stage PCR (qPCR) workflow (Quick-16S NGS Library Prep Kit, Cat.no.:6400, Zymo Research), enabling both amplification and direct quantification of PCR products. The protocol for preparing 16S Metagenomic libraries was as recommended by the manufacturer (Amplicon PC, Clean-Up PC, Index PC. 16S Metagenomic sequencing library preparation. Illumina: San Diego, CA, USA. 2013). The workflow of this assay consists of two separate PCR reactions (both of which do not include specific or unspecific fluorescent probes) to amplify the genome region of interest for the follow-up genome sequencing. Results obtained from this process are then used to calculate the relative abundance of the genera/species present in a sample. The first qPCR incorporated V3-V4 primers to target the conserved regions of the 16S rRNA gene employing a 25-cycle program: 95 °C for 3 min followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for another 30 s, before heating up to 72 °C for 5 min and holding at 4 °C. In this work, a focus was set on the bacterial community only, because among archaea no hygienically relevant representatives have been documented so far (Cavicchioli et al., 2003). After enzymatic clean-up, a second PCR using index primers (Quick-16S NGS Library Prep Kit, Cat.no.:6400, Zymo Research) was performed to attach barcodes and flow cell adapters, following a protocol of initial denaturation at 95 °C for 1 min and 5 cycles of 95 °C for 30 s, 55 °C for 30 s, 55 °C for 30 s, 72 °C for 3 min, before final extension at 72 °C for 5 min and holding at 4 °C. After pooling and final clean-up, libraries were quality-controlled and quantified. Ready-to-sequence libraries were sequenced on an Illumina platform using 2 × 300 bp paired-end sequencing. All samples yielded more than 50′000 reads, were then demultiplexed and Illumina adaptor residuals trimmed. A 'no template control' (NTC) in which the template DNA was replaced by

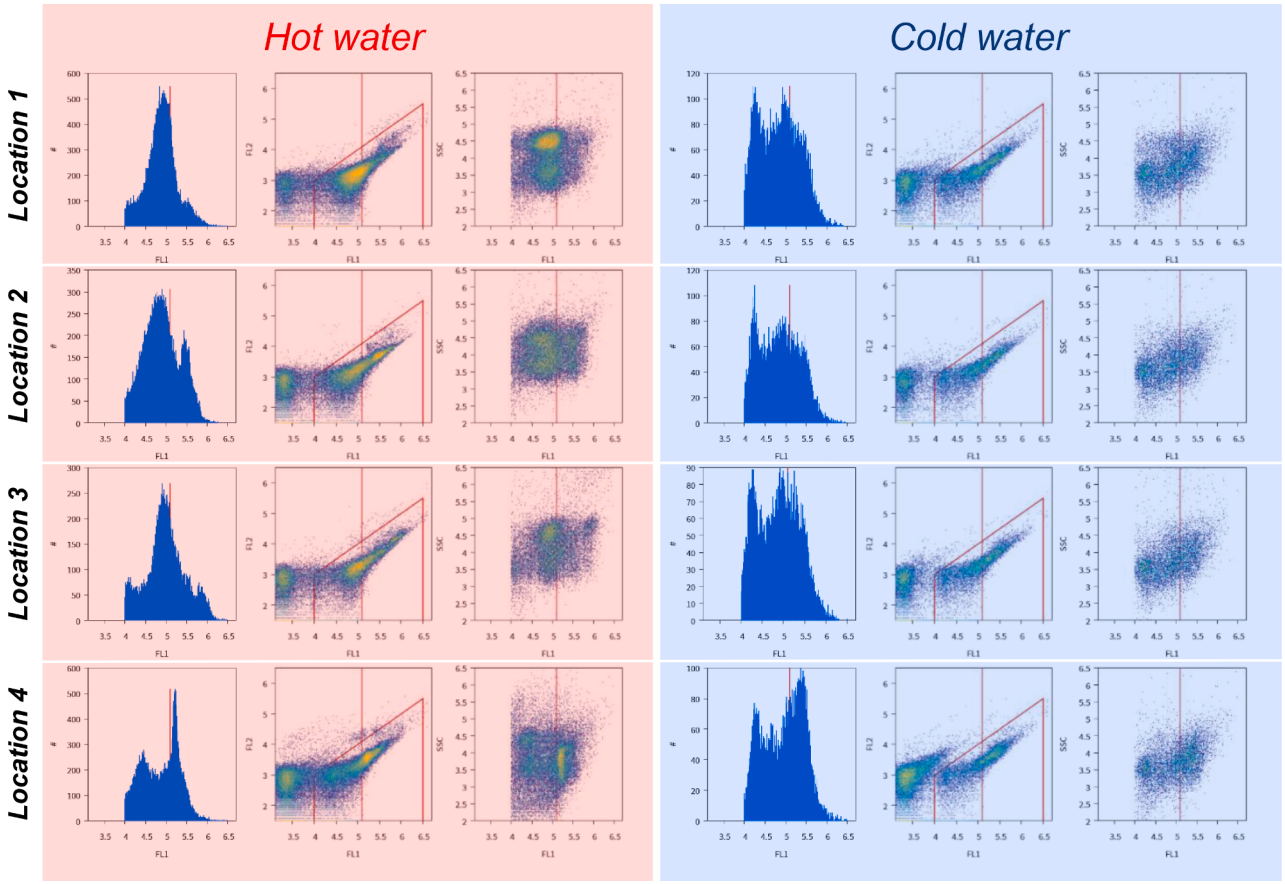

Fig. 2. FCM-fingerprints of cold- and hot-water samples collected from neighbouring buildings supplied from the same drinking water supply pipe (locations 1–3), and of a building (location 4) located in a different part of the municipality but supplied from the same public drinking water distribution network. Samples from locations 1–3 were collected on May 6, 2022, between 7am and 9am, cooled down to 4 °C and analysed within 2 h with BactoSense TCC. Samples from location 4 were collected on May 8.
TCC concentrations at locations 1 - 4 in cold water were: 131'030 cells/mL, 106'320cells/mL, 110'210 cells/mL, 108'010 cells/mL, respectively; in hot water: 405'550 cells/mL, 323'150 cells/mL, 225'930 cells/mL, 360'420 cells/mL, respectively.

molecular grade water was included to assess cross-contamination throughout library preparation. All identified taxa in the NTC (*Escherichia-Shigella, Aquabacterium, Moraxella, Paracoccus, Cutibacterium*) were removed from the sequenced samples.

2.8.2. Raw reads quality control

Raw reads were checked using FastQC and quality filtered/trimmed using cutadapt v3.0 (see Table 20,221,115_16S_profiling.xlsx sheet 'raw_reads_qc' (Supplementary Materials, Table S3)). Samples with more than 250 000 read pairs were down-sampled to that value.

2.8.3. 16S rRNA-gene-profiling

For 16S rRNA-gene-profiling, sample processing started by trimming the adapter sequences using cutadapt v3.0, followed by read length thresholding. The DADA2 (1.18.0) pipeline was used to construct the amplicon sequence variant (ASV) table, a higher-resolution analogue of the traditional operational taxonomic unit (OTU) table. Taxonomic classification was determined using the SILVA rRNA reference database v138. Cross-contamination occurring throughout library preparation was not detected.

A complete list of detected bacteria can be found in Supplementary Materials, Table S3. The SILVA rRNA reference database v138 contains additional classifications to the NCBI taxonomy, including phylogenetically coherent groups above the family rank, consisting only of sequences from uncultured organisms.

2.9. Community analysis

All obtained ASVs were compared to sequences in the TEMPURA database for usual and rare prokaryotes (Sato et al., 2020) and assigned as potentially thermophilic.

3. Results

3.1. FCM analysis of water samples collected from DWI

3.1.1. TCCs and FCM-fingerprints of hot- and cold-water samples

Commonly, 2- to 4-fold higher TCC concentrations ($TCC_{h/c}$-range: 1.11 - 8.74) were observed in hot water collected from boilers compared to those in the supplied cold DW (Table 2). Interestingly, in communities where DW was produced mainly from ground- and spring-water, TCC concentrations in cold water were very low (< 50'000 cells/mL; locations 7, 8, 11, 16, 17, 19), and in most of them (7, 11, 16, 17) only slightly enhanced TCCs were observed in the corresponding hot-water ($TCC_{(h/c)} < 1.5$). In contrast, in hot water from locations 8 and 19 almost 9-fold higher TCC concentrations were found, suggesting regrowth in the installation.

Distinctly differing FCM-fingerprints for cold and hot water were observed at all locations. Visually, differences are recognized best when comparing FL1-SCC plots (Fig. 1; Supplementary Materials, Fig. S3). Distinct cell clusters are obvious in most hot-water samples. Their positions, numbers and density differ clearly from the (less distinct) clusters observed in corresponding cold-water samples. In samples with low

TCC concentrations in cold water (7, 11, 16, 17) such patterns are less obvious. The remarkable differences in cluster patterns suggest growth and enrichment of specific microbial communities in hot water prepared in boilers. Often, cell clusters could not be clearly attributed to so-called LNA- or HNA-regions (e.g., Fig. 1, location 5), which are frequently used to characterize FCM-fingerprints (Egli and Kötzsch, 2015); therefore, we do not use this parameter for fingerprint characterization here.

Results from locations 1–3 are particularly interesting (Fig. 2). The three buildings, located not more than 60 m from each other, are connected to the same communal DW supply pipe. Whereas TCCs and fingerprints from the three cold-water samples are virtually identical, those of the hot-water samples differ, firstly, from those of the cold water, and secondly – more importantly – amongst each other. This suggests that each of the boilers contained its individual microbial hot-water community, even when fed with the same cold water. Also included in Fig. 2 are results from location 4, a building supplied with DW from the same treatment facility but situated in a different area of the community. Although sampled two days later, cold-water TCC concentration and FCM-fingerprint resemble those found at locations 1–3, however, the hot-water fingerprint is again clearly different.

Location 18 provides a control, as hot water is prepared here with a flow-through heater system. As expected, in the hot water produced the TCC concentration was slightly reduced compared to the supplied cold DW (Table 2). However, SCC-FL1 dot plots revealed little difference between cold and hot water (Fig. 1, Supplementary Materials, Fig. S3).

3.1.2. *Bray-Curtis (BC) dissimilarity analysis of FCM-fingerprints*

To evaluate the extent of visually obvious differences of FCM-fingerprints of the incoming cold DW and the hot water produced thereof in boilers, we performed a PCA projection of the FCM-fingerprint (Fig. 3) as well as a beta diversity analysis on FCM fingerprints using the BC dissimilarity metric (see Section 2.5). In Fig. 3, samples harbouring similar microbial traits are positioned closer to one another.

The ANOSIM statistical analysis revealed a significant separation between cold-water and hot-water fingerprints into two distinct populations ($R = 0.47$, $p = 0.01$, with 100 permutations). The pairwise dissimilarity between hot- and cold-water fingerprints is detailed in Supplementary Materials, Table S2. On average, the BC dissimilarity between hot- water and cold-water samples was 0.34, with notable outliers being samples 12 and 18. The latter was our control sample, not from a boiler but a flow-through heater system, therefore, it is expected that bacterial communities are similar. The inter-class dissimilarity was calculated as 0.34 for hot, and 0.24 for cold water, implying that cold water-samples exhibit more similar fingerprints, while hot-water samples exhibit higher population diversity. Nevertheless, when comparing replicate measurements from the same water source, the BC dissimilarity was, on average, 0.06 lower in hot-water compared to cold-water replicates. This observation suggests that, within one water type, bacterial communities in boilers tend to form a more consistent subset of bacterial types, whereas bacterial populations in cold water can exhibit greater variability.

For the identification of hot water bacterial populations in unknown samples, we developed a Support Vector Machine (SVM) classifier using 94 FCM fingerprints after PCA dimensionality reduction to the first 20 principal components, excluding samples from location 18 due to their origin from a flow-through heater. Employing a leave-one-out cross-validation approach, our SVM classifier achieved a classification accuracy of 0.97, corresponding to a 3 % error rate. Samples 7h and 12h were misclassified, while other samples classified as outliers in Fig. 3, such as 4h, 11c, and 13c, were successfully classified by the SVM using information from other PCA components of the fingerprint.

3.2. *Growth properties of microbial cold- and hot-water consortia*

The throughout enhanced TCCs in hot water compared to those in the supplied cold DW (Table 2, Figs. 1, 2) imply microbial growth in boiler water. Heating of cold DW appears to make nutrients available for growth of a – presumably thermophilic – microbial community. In *ex-situ* batch experiments, we tested whether observed growth resulted from a quick activation of thermophilic strains present in the cold DW, or from a hot-water-specific thermophilic microbial community. For this, cold DW was heated to 55 °C and kept at this temperature for some 12 h, either as is (control), or with ~1/3rd (v/v) of hot water added. An example for an assay amended with hot water is shown in Supplementary Materials, Fig. S4a, where a batch growth pattern with exponential increase of TCC concentration and a stationary phase was recorded. Deduced doubling times were in the range of 12–13 h for the whole population, and approximately 7 h, assuming only cells originating from hot water were able to proliferate (see Supplementary Materials, Fig. S4b).

To extend the range of batch growth for the bacterial hot-water inoculum, assays were modified so that most of the (obviously non-growing, but background-generating) cold-water cells were removed by 0.2 μm-filtration, and the filtered cold water was used as a growth medium. Such assays were inoculated with 15–20 % (v/v) of unfiltered either cold or hot water. The results for two assays incubated at 53 – 54 °C performed in autumn 2020 with water from location 1 is depicted in Fig. 4. Whereas no significant growth was observed in the assay amended with cold DW, cell proliferation started immediately and accelerated with time in the assay inoculated with hot water. Initially, t_d for the hot-water flora was estimated to be in the range of 11 h, later it reached 4.5 h (Supplementary Materials, Fig. S5). A similar experiment was performed in spring 2022, after the community had introduced UVC treatment as a last step before distribution. Also here, growth was recorded at 54 °C in assays consisting of 90 % 0.2 μm-filtered cold and 10 % hot water, indicating that the thermophilic microbial community in this boiler does not depend on being fed with alive cold-water cells (Supplementary Materials, Fig. S6). Similar batch growth experiments were also performed with water collected from locations 4 and 5. Also here, insignificant growth was detected for assays inoculated with cold water, whereas hot water-inoculated assays exhibited typically doubling times between 4 and 8 h (results not shown). Clearly, the results demonstrate that hot water from boilers contains an active thermophilic microbial flora that can immediately grow in DW heated up to boiler temperatures. No growth temperature optimum for a hot-water community from a particular location was determined, however, growth was consistently observed in assays incubated between 50 and 60 °C. Still, at

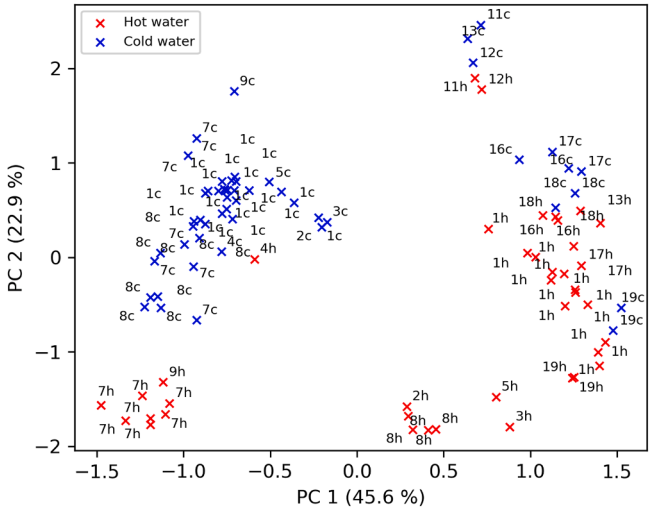

Fig. 3. Two-dimensional projections of bacterial population fingerprints using PCA (first two principal components). Each blue and red dot represents a sample from cold and hot water, respectively. On the axes, the first and second PCA principal components and their explainable variance are displayed (for more information see Table S2).

an incubation temperature of 58 ± 1 °C shortest recorded doubling times ranged from 5 - 10 h (results not shown). In contrast, neither hot- nor cold-water-inoculated batch assays incubated at room temperature (20 - 30 °C) exhibited significant growth within the incubation time tested, typically 12 - 14 h (Supplementary Materials, Fig. S7).

3.3. Composition and diversity of bacterial cold- and hot-water communities

At the level of Amplicon Sequence Variations (ASV), notably more diverse communities were observed based on their alpha diversity in cold-DW boilers than in their hot-water counterparts (Fig. 5A). With 287 unique ASV, cold-water samples showed an almost 1-log higher diversity than their hot-water counterparts (34 unique ASV), while 311 ASV could be found in both microbiomes (Fig. 5B). Of the taxa identified as unique to hot-water samples, the majority (58.3 %) was previously described as thermophile (i.e., able to reproduce at temperatures above 25 °C) according to the TEMPURA database of growth temperatures of usual and rare prokaryotes (Sato et al., 2020). For taxa unique to cold-water samples, the percentage was only 13.6 %.

A more diverse community composition in cold water was observed throughout the whole sample set, independent of location (Fig. 5A). The community compositions in cold-water samples were distinctly different from their hot-water counterparts and both water types formed distinct clusters in the Principal Coordinates Analysis (PCoA) plots (Fig. 6), specifically when cold waters supplying the hot-water boilers were directly compared to each other.

Hydrogenophilaceae dominated in virtually all hot-water samples, independent of sampling location (Fig. 7). In addition, *Nitrosomonadaceae* and *Thermaceae* were present at high relative abundances in most hot-water samples, respectively. Specifically, the thermophilic species *Thermus scotoductus* was among the three most abundant taxa in 8 out of 12 investigated hot-water boilers (Supplementary Materials, Table S3). High relative abundances were also found for *Vicinamibacterales* in six hot-water systems and for *Pyrinomonas* at site 19, despite their absence from cold-water samples. *Rhodoferax, Polaromonas* (both *Comamonadaceae*), candidate order *Peribacteria*, family SM2D12 (order *Rickettsiales*) and genus IS-44 (family *Nitrosomonadaceae*) were identified as the relatively most abundant taxa in the cold-water samples (Fig. 7). The significantly higher number of ASV unique to cold-water microbiomes in comparison to hot water resulted in a higher richness and evenness of genera and taxa (Supplementary Materials, Fig. S8, Table S3). *Nitrospira, Bdellovibrio*, and *Reyranella* were present in all cold-water samples. *Legionellae* were present at nearly all sampled locations (except location 2) but were not specifically enriched in hot-water boilers. They were found in all cold-water samples with a maximum proportion of 0.79 % of the bacterial community, while they were present in half of the hot-water samples with a maximum proportion of 0.37 % (Supplementary Materials, Fig. S8, Table S3). Other opportunistic pathogen taxa commonly associated with DW distribution systems such as *Mycobacterium* or *Pseudomonas* were only detected in a few isolated samples at low proportions (Supplementary Materials, Table S3).

While hot and cold-water samples showed distinct bacterial community profiles, no differences were observed in cold and hot water heated with the flow-through heater used as a control (Figs. 5–7). These samples were dominated by representatives of *Methylophilaceae* and *Comamonadaceae*.

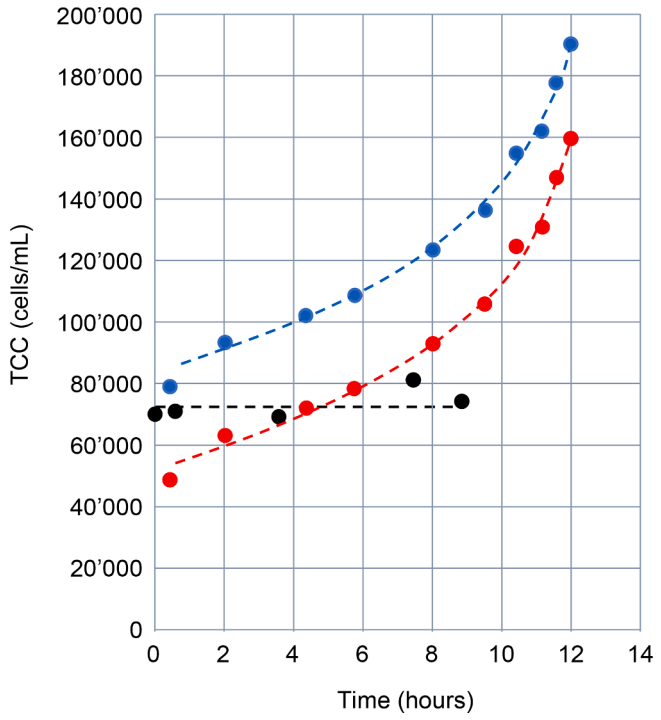

Fig. 4. Batch growth experiments performed with drinking water from location 1. The assay consisted of 50 mL of 0.22 µm-filtered cold-water heated up to 54 °C and inoculated with 10 mL of hot water (blue dots for total flora; red dots for hot-water flora). The control assay contained 50 mL of 0.22 µm-filtered cold-water of, heated up to 54 °C and was inoculated with 10 mL of cold water (black dots). Subsequently, assays were incubated in a water bath at 53±1 °C. No clear exponential phase was observed, doubling times ranged from approx. 11 h (between 2- 8 h incubation time), and approx. 4.5 h (between 10 - 12 h incubation time). Experiment performed October 27, 2020.

4. Discussion

4.1. FCM total cell concentrations, fingerprints, AOC generation and microbial growth in DHWIs

4.1.1. Flow cytometry for characterizing DWIs

As far as we are aware, this is the first report documenting consistently increased TCCs in hot water from boilers in private and public buildings, compared to concentrations found in the supplied cold DW. Two earlier studies reported microbial cell concentrations in cold and hot water in buildings (Henne et al., 2013; Zacheus and Martikainen, 1995). Furthermore, Ley and colleagues recently documented elevated concentrations of both cultivable (heterotrophic plate counts) and total cell concentrations in hot-water samples from a residential building supplied with chlorinated DW (Ley et al., 2020).

FCM proved an excellent tool for rapidly characterizing DWIs with respect to quantity and fingerprints of microbial flora in cold and hot water. A set gate could be used for determining TCC in all samples, hot and cold. However, reliable allocation of clusters to small (LNA) and large (HNA) cells, as commonly observed in natural waters and DW (Gasol et al., 1999; Hammes and Egli, 2010; Hammes et al., 2008; Koch et al., 2014), was not possible as positions of cell clusters shifted more freely. Here, a DNA-staining-method for total bacterial cells (alive and dead) was employed, but applying viability and activity stains might produce additional, different information.

4.1.2. Thermophilic microbial growth in DHWIs

TCCs in cold DW > 100′000 cells mL^{-1} and $TCC_{(h/c)}$-ratios in the range of 2–3 were usually observed in communities that used lake water to produce DW. At location 1 this ratio was surprisingly stable over a period of two months (Supplementary Materials, Fig. S9). In communities preparing DW primarily from groundwater, the ratio of $TCC_{(h/c)}$ was in the range of 1.5 and TCCs were < 50′000 cells mL^{-1}. This suggests that raw water quality might influence both, TCC concentration in the resulting cold DW, as well as that in hot water prepared thereof in boilers. Clearly, the relationship between content and composition of DOC and AOC generated upon heating of cold DW deserves further

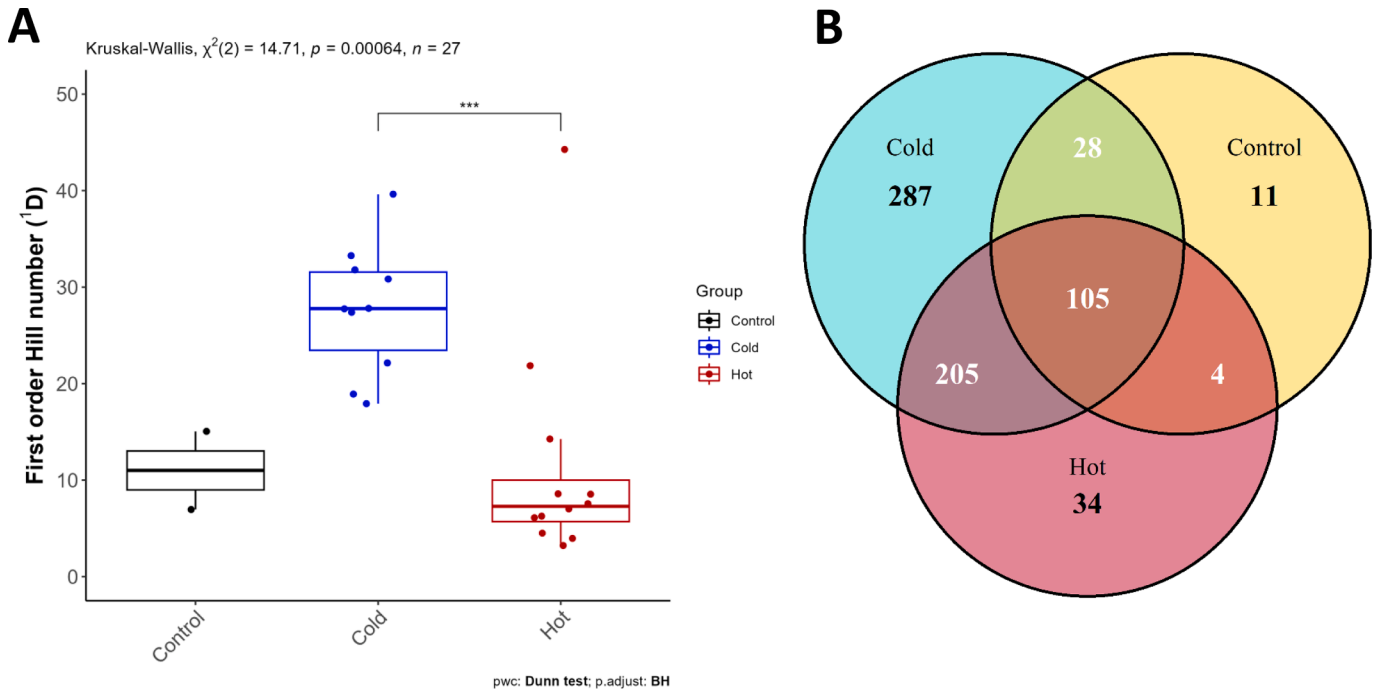

Fig. 5. First order hill number depicting the alpha diversity of bacterial community composition and calculated for amplicon sequences unique to hot, cold and control sampling sites (flow-through heater samples 18h and 18c). (A) shows a significantly more diverse community composition in cold-water samples. Cold-water microbiomes have also been observed as having the highest number of unique sequences (287) while 205 are present in both hot- and cold-water samples. (B) Merely 34 unique ASV were only detectable in hot-water boilers and 11 in the experimental control, the flow through-heater installation.

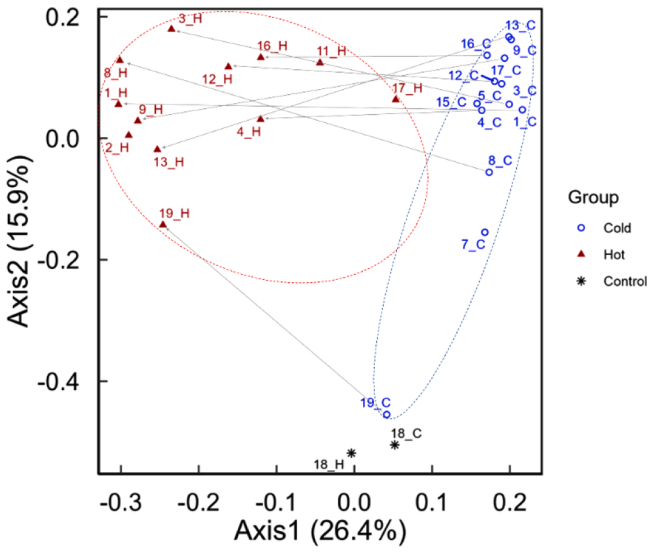

Fig. 6. PCoA plot depicting the relative differences of microbial community composition in samples obtained from the 14 locations and the experimental control (location 18) from supplied cold water and hot water from corresponding boilers. Samples collected from locations with the same supply of cold water (locations 1–3) show a significant (p-value ≤ 0.0001) and clear shift in hot water from respective boilers. *, Cold- and hot-water samples from flow-through heater installation (control). Note that samples 2c, 5h, 7h and 11c do not exist (DNA amplification not successful).

attention.

Inoculated into cold DW heated up to 50 - 60 °C, hot-water flora grew immediately with doubling times as short as 4 - 8 h, whereas cold-water flora was unable to proliferate under such conditions. This, and the high RNA content of microbial hot water flora reported by Henne et al. (Henne et al., 2013), suggests that hot-water boilers are semi-continuous flow-through bioreactors. Using long-term batch assays, similar observations were made recently (Meyer et al., 2023), namely, regrowth of microbial hot-water flora at 50 – 60 °C but not at 22 °C (and *vice-versa* for cold-water flora) during incubation of water samples for three weeks at different temperatures. However, many aspects still remain to be investigated, e.g., effects of temperatures between 30 - 60 °C on composition of hot-water flora, on growth yields and kinetics, or on contributions from suspended cells and from biofilms (Bagh et al., 1999; Preciado et al., 2021). Such information is essential, not only for DWIs using boilers, but also for hot climate where water storage containers and installations encounter temperatures between 30 and 50 °C.

Notably, also boilers receiving UVC-treated cold DW with inactivated microbial flora (presumably supplemented with some detached viable cells from pipe biofilms), contained an active, thermophilic microbial flora. *Ex-situ* batch growth assays with hot-water flora from location 1 exhibited similar growth, before and after introducing UVC-treatment in this community. This confirms boilers as separate, thermophilic microbial ecosystems that are quite resistant to external perturbations. The presence of an active hot-water flora has been documented repeatedly also in DHWIs fed with chlorinated DW (Henne et al., 2013; Ley et al., 2020; Zacheus and Martikainen, 1995).

4.1.3. *Possible mechanisms of AOC generation supporting thermophilic growth in boilers*

In DW heterotrophic microbial growth is typically limited by AOC availability (Hammes and Egli, 2005; Lautenschlager et al., 2014; Van der Kooij, 2002). Sources of AOC might be i) hydrolysis of DOC, ii) lysis of cold-water flora, or iii) migration of AOC from material's surfaces. The amount of DOC hydrolysed that would be necessary to support the observed TCC increase in hot water can be estimated from established cell yields for growth of natural microbial flora on AOC (Hammes and Egli, 2005). Choosing locations 1–4 as an example (all supplied with DW produced mainly from lake water containing between 1 – 1.5 mg L^{-1} of DOC), the increase of TCCs in hot water ranged from ca. 100′000 – 300′000 cells mL^{-1} (Table 2). Adopting an average cell yield from AOC of 10^7 cells μg^{-1} (Hammes and Egli, 2005) implies that generation of 10

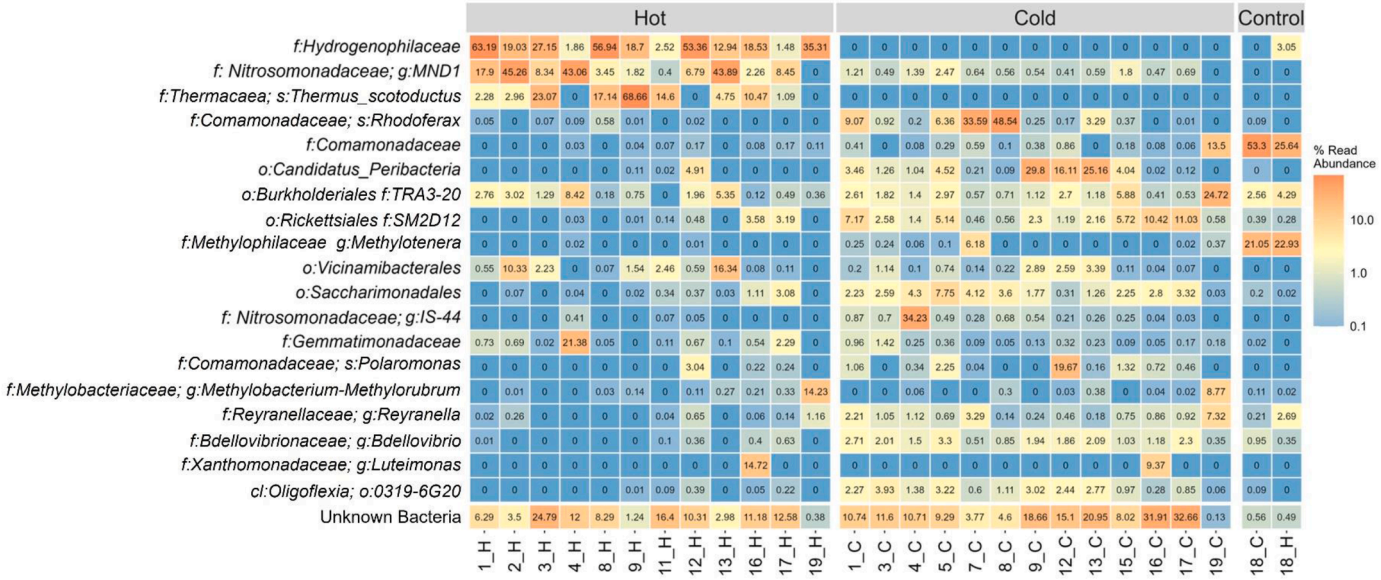

Fig. 7. Heat map of the relatively most abundant prokaryotic taxa in hot- and cold-water samples in% of read abundance. Detailed information on locations and samples are listed in Table 1 and Table 2. Note that amplification of DNA from samples from locations 2c, 5h, 7h and 11c was not successful. # 6, 10, and 14 were used as negative controls for DNA extraction and are therefore absent. Samples from location 18 were collected directly before and after a flow-through heater system and is therefore referred to as 'control'. Location #15 represents a sample from the treatment plant supplying locations #1–4, therefore, only a cold-water sample exists.

μg AOC L^{-1} during heating should result in an increase of 100′000 cells mL^{-1}. Assuming a typical DOC concentration of 1 mg L^{-1} in cold DW, this would correspond to hydrolysis of 1 – 3% of DOC only. Such small differences are analytically difficult to quantify. Earlier reports on microbial regrowth during overnight stagnation in buildings came to similar conclusions (Lautenschlager et al., 2010). Alternatively, it was suggested that heat-induced lysis of cells entering the boiler with cold DW might be the source of AOC for growth of the hot-water flora (Henne et al., 2013; Ji et al., 2017). However, assuming an average single cell in DW flora to consist of 2×10^{-14} g of carbon (Hammes and Egli, 2005), complete lysis of 100′000 cells mL^{-1} would result in 2 μg of C L^{-1}, allowing the formation of 20′000 cells mL^{-1}, at the most. Yet, in aerobic thermophilic sludge digestors only some 30 % of cell lysis products were bioavailable (Mason et al., 1986). Furthermore, cell concentrations in batch growth experiments inoculated with cold water flora indicated little cell lysis after heating to 55 °C (Fig. 4, Supplementary Materials, Fig. S6). Another alternative explanation for the observed thermophilic growth could be the migration of AOC from boiler and pipe surfaces into the bulk water phase (Bucheli-Witschel et al., 2012; Wen et al., 2015), or from biofilms (Pick et al., 2021). However, the results in Table 1 indicate no obvious relationship, neither between S/V-ratio, nor age of boilers, and extent of thermophilic growth in hot water. The smallest boilers with the largest S/V-ratios exhibit only little thermophilic growth ($TCC_{(h/c)}$ ~1.5), whereas for larger boilers with less favourable S/V-ratios more extensive growth ($TCC_{(h/c)}$ between 3 and 9) was observed. This makes a release of AOC from surfaces or biofilms less likely.

Hence, the most likely source of AOC for growth of the hot-water community appears to be (bio)chemical DOC hydrolysis due to heating. In fact, the extent of cell growth in hot water and the $TCC_{(h/c)}$-ratio might reflect the amount and quality of DOC present in the supplied cold DW and its stability when heated up to boiler temperatures. Results obtained from location 1 where a stable ratio of $TCC_{(h/c)}$ of ~3.8 was observed over a period of two months (Supplementary Materials, Fig. S9) suggest that this parameter has considerable potential for the characterization of DWIs. For example, the ratio might be used as a quick and rough indication of DOC stability, and, perhaps, biostability of a DW after production in general. Most likely, the extent of thermophilic growth will depend also on refill patterns and turn-over-times of hot water in boilers. Presently, no information appears to be available in the literature, neither concerning physical nor (bio)chemical mechanisms and kinetics of the amount and quality of AOC generated from DOC hydrolysis during hot water production, nor its role in the selection and establishment of different thermophilic floras in boilers and other hot-water installations. Nevertheless, the batch growth experiments described here suggest that AOC generation is fast and almost completed within 10–12 h.

4.2. Bacterial communities in DWIs

Despite the lower bacterial diversity found in hot-water samples they generally exhibited a higher cell concentration than their cold-water counterparts. Considering heat an ecological stress that clears previously occupied niches, competitive pressure on the surviving and adapted taxa is lower. Similar observations were made in hot springs as the result of an optimization between using all possible nutrients and filling all available niches while minimizing metabolic overlaps (Escuder-Rodríguez et al., 2022). Transient availability of AOC, e. g., after refilling of the boiler, may select for fast-growing organisms that out-compete others, ultimately limiting diversity (Proctor et al., 2017).

Previous studies (Henne et al., 2013; Zhang et al., 2021) suggested that only a small percentage of DW bacteria can adapt to high temperatures, with only few taxa being able to survive the stressor heat that exerts a strong selection pressure, such as the need for thermostable membranes and enzymes. Similarly, results from other DHWI (Meyer et al., 2023) suggest the existence of heat-adapted bacterial populations with lower taxonomic diversity. Of all specific taxa identified in the DHWIs here, 58 % were previously described as having thermophilic properties according to the TempURA database (Sato et al., 2020). Most likely, the ability to thrive at temperature above 45 °C is a considerable driver in their bacterial community composition, a trend previously described for hospital water systems and high temperature environments such as hot springs (Ji et al., 2018).

Shortly after the isolation of the first non-spore-forming Gram-negative thermophilic bacteria from natural hot springs, the presence of bacterial cells in DHWI, both boilers and hot-water distribution pipes,

was observed (Brock and Boylen, 1973; Pask-Hughes and Williams, 1975; Stramer and Starzyk, 1981). Most of these isolates resembled the thermophilic bacterium *Thermus aquaticus*. In 2019, Wilpiszeski and colleagues described the Gram-negative thermophilic bacterium *Thermus scotoductus* in domestic water heaters throughout the US. The relative dominance of this taxon in most samples analysed here (9 out of 13; regardless of the origin of the source water), is therefore not surprising, especially considering that its members have been described as well adapted for nutrient-poor conditions as commonly found in DW (Gounder et al., 2011).

Other members of the hot-water communities included *Nitrosomonadaceae, Hydrogenophilaceae* and *Vicinamibacterales* strains, all of which have been widely reported in comparable studies in the past (Ji et al., 2017). With our focus on DWIs, the presence of microorganisms relevant for consumer safety, i.e., obligate or opportunistic pathogens, were of special interest. Although communities in high temperature boilers were dominated by Gram-negative bacterial taxa, common pathogenic genera associated with hot water (namely *Legionella pneumophila*) were rarely observed. This could be due to the inherent limitations of the 16S rRNA amplicon sequencing method (insufficient resolution) and the tendency of prokaryotic genome libraries to be dominated by clinical strains, leaving environmental samples not adequately addressed (Bharti and Grimm, 2021). However, *Legionella* growth may have been effectively prevented in the DHWIs sampled in here, because most of the boilers had water temperatures above 55 °C (Table 2). Such temperatures not only inhibit *Legionella* proliferation, but are also restricting growth of *Legionella*-harbouring amoeba that inhabit pipe wall biofilms (Ahmad et al., 2021).

When compared to the hot-water samples, the bacterial communities in the cold-water systems were much more diverse with many common groundwater taxa shared between all sites (despite geographic differences of the sampling locations). Furthermore, a significant number of taxa were found in both cold- and hot-water samples, which indicates an (at least temporary) ability to endure both conditions. Previous work has shown the presence of a baseline level of thermophilic bacteria like *Legionella* spp. that persisted under adverse conditions and started to proliferate and colonize ecological niches opened-up by changes in temperature in a simulated DW distribution system (Shaheen et al., 2019). Similar behaviour could explain the minimal concentrations of (most likely planktonic) *Hydrogenophilaceae* and *Nitrosomonadaceae* in cold water and their dominance in hot-water samples. Due to the inaccessibility of the DHWI investigated here biofilm samples were not collected and the focus was put on the bulk water phase. While previous studies investigating comparable systems (Ji et al., 2015; Ji et al., 2017) indicated that biofilm appears to have little influence on the composition of the bacterial community in DW, other reports suggest the opposite (Bagh et al., 1999; Preciado et al., 2021). Follow-up studies might include aspects of biofilms to gain better insights into their contribution in shaping the entire DW microbiome, both suspended and attached.

4.3. FCM-fingerprinting versus microbial community analysis

Recently, significant advances have been made in the analysis and application of flow cytometric data. Cell sorting, subsequent sequencing of isolated clusters, and analysis with advanced mathematical tools have allowed to produce links between FCM-fingerprints and 16S rRNA-gene-based microbial community composition (Heyse et al., 2021; Rubbens and Props, 2021). FCM-derived diversity metrics were shown to correlate well with sequencing-derived information in aquatic environments (García et al., 2015; Heyse et al., 2021; Proctor et al., 2017; Props et al., 2016; Rubbens et al., 2021).

Obviously, our data demonstrate that TCC-based FCM-fingerprints provide fast and reliable information on changes of microbial communities in domestic water installations, not only with respect to cell density, but also with respect to composition. In both, FCM-fingerprinting and community analysis, water samples cluster closely when they are expected to contain identical or similar microbial communities (Figs. 3 and 6). Indeed, after appropriate training, FCM-fingerprint analysis can distinguish between cold- and hot-water samples with an accuracy of 97 %. Despite this, presented data do not allow to draw conclusions concerning the specific composition of the microbial communities and/or their metabolic capabilities. Linking information produced with the two methods would need additional data and deeper analysis of FCM-fingerprints, including isolation of cells from clusters and their characterization (Heyse et al., 2021; Koch et al., 2014; Rubbens and Props, 2021). However, investigation of DWIs as documented here might prove a good field for exploring links between FCM and sequencing information.

4.4. Outlook

Interactions and dependencies between harmless (even useful) microbiomes, opportunistic pathogens, nutrient availability, bulk water, and biofilms in drinking water distribution pipes is an ongoing debate (see e.g., (Ji et al., 2017; Logan-Jackson et al., 2023; Pick et al., 2021)). The fact that consistently enhanced cell concentrations were detected in hot-water samples from boilers, is most likely due to increased availability of AOC. Higher (bio)chemical DOC-hydrolysis rates at increased temperatures according to Arrhenius' law, should result in higher amounts of AOC generated per unit of time, and, in an AOC-limited batch system, should enable additional microbial growth. It remains to be tested whether increased rates of (partial) lysis of temperature-labile cells from cold DW or leaching of nutrients from biofilms and surfaces add to this process. Such generation of AOC will not only allow growth of the observed thermophilic microbial communities but might also support (necrotrophic) growth of *Legionella* spp., as described earlier (Dai et al., 2018; Temmerman et al., 2006; Van der Kooij et al., 2017).

In general, growth of microbial communities during distribution of (non-disinfected) DW in public distribution systems and DWIs and their relationship with temperature and AOC, may be outlined as follows: At temperatures below 15 °C little (re)growth is usually observed (Lautenschlager et al., 2013; Van der Kooij and van der Wielen, 2014) with a high diversity of the psychrotrophic/mesophilic microbial community. Above 15 °C (re)growth of mesophilic microbial communities is commonly observed during distribution, particularly during long residence times and stagnation. This (re)growth relies on strains from the alive mesophilic microbial community present in DW and biofilms. Above 50 °C, temperature as a stressor appears to take over and a shift from mesophilic to thermophilic microbial growth occurs. In this temperature range growth of the microbial community is not anymore dependent on a selection of strains from incoming active flora in the cold DW but relies on a separate, established (boiler-specific) thermophilic microbial community. This transition could cause the drastic reduction of community diversity observed here. Similarly, Ji and colleagues proposed a shifting point for the transition of phylogenetic functions and composition of microbiota from mesophilic to thermophilic conditions at 51 °C (Ji et al., 2017). How this might be at temperatures above 60–70 °C, where not only temperature but also chemical precipitation processes determine the availability of inorganic nutrients like nitrogen, phosphorus etc., remains to be investigated.

Also, in view of the present discussion of energy saving versus *Legionella* prevention, a better understanding of microbiomes existing in hot- and warm-water installations in buildings is needed. This should include not only installations that are maintained between 50 and 60 °C but also DW storage tanks that are kept or heat-up to temperatures between 30 and 50 °C. The reported wide geographical occurrence of

strains of *Thermus* (Brock and Boylen, 1973; Ji et al., 2017; Wilpiszeski et al., 2019); this report) in both DW distribution systems with or without disinfection residual, suggest that the phenomenon of thermophilic communities in DHWIs is the rule rather than the exception. Its hygienic relevance remains to be studied.

5. Conclusions

- Flow cytometric total cell counting and fingerprint analysis allowed rapid detection of changes of abundance and composition of microbial floras in domestic cold- and hot-water installations.
- At 14 domestic water installations using boilers to prepare hot water, hot water always contained higher TCC concentrations than the supplied cold DW, and FCM-fingerprints and microbiome composition of supplied cold DW were very different from those in hot water.
- The phenomenon was not transient, but the same FCM-pattern was observed at several locations over a 4-year period.
- Heating of cold DW to boiler temperatures leads to the formation of nutrients, most likely AOC.
- Hot water in domestic boilers contained a thermophilic microbiota able to grow at 50–60 °C with doubling times between 5 and 10 h, whereas microbial flora in the supplied cold DW was unable to grow at such temperatures.
- Microbial floras in boilers appeared to develop individually; even if supplied with the same cold DW, different boilers contain very different microbial communities.
- In both, diversity analyses of FCM-fingerprints and bacterial community composition, cold-water samples clustered separately from hot-water samples and cold DW samples showed an almost 1-log higher bacterial diversity than corresponding hot-water samples.
- Gram-negative taxa *Hydrogenophilaceae, Nitrosomonadaceae, Thermaceae* dominated in virtually all hot water samples, whereas *Rhodoferax, Polaromonas* (both *Comamonadaceae*), candidate order *Peribacteria*, family SM2D12 (order *Rickettsiales*) and genus IS-44 (family *Nitrosomonadaceae*) were identified as the relatively most abundant taxa in the cold-water samples.
- Boilers and hot-water installations contain a so-far neglected thermophilic microbial ecosystem the hygienic relevance of which remains to be investigated.

CRediT authorship contribution statement

Thomas Egli: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Lena Campostrini:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. **Mats Leifels:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Hans Peter Füchslin:** Data curation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing. **Claudia Kolm:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Cheng Dan:** Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Stefan Zimmermann:** Data curation, Formal analysis, Resources, Writing – original draft, Writing – review & editing. **Vivian Hauss:** Data curation, Formal analysis, Methodology, Resources, Writing – original draft, Writing – review & editing. **Alexandre Guiller:** Data curation, Formal analysis, Resources, Writing – original draft, Writing – review & editing. **Luigino Grasso:** Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Writing – original draft, Writing – review & editing. **Adrian Shajkofci:** Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. **Andreas H. Farnleitner:** Data curation, Formal analysis, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Alexander K.T. Kirschner:** Data curation, Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Thomas Egli reports financial support, article publishing charges, and equipment, drugs, or supplies were provided by bNovate Technologies SA. Thomas Egli reports financial support, administrative support, and article publishing charges were provided by Microbes-in-Water GmbH. Thomas Egli reports a relationship with bNovate Technologies SA that includes: equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

We thank the following persons for support in various ways: R. Bixa, K. Egli, I. Fritz, H.-P. Greb, C. & B. Herzog, B. & J. Hunn, T. Meier, A. Mustedanagic, U. Schär, G. Schenk, and J. Senouillet. C. M. Egli is acknowledged for careful reading of the various drafts. We also thank two anonymous reviewers for valuable suggestions.

Disclosure declaration

No AI-assisted technologies were used in writing this manuscript.

Contributors' declaration

Contributions of individual authors were: original concept and planning (TE), confirmation sampling campaign (TE, LC, HPF, SZ, LG, AF, AK), first sampling campaign (TE, LC, HPF, SZ, VH, AG, LG, AK), second sampling campaign (TE, LC, HPF, SZ, VH, AK), FCM analysis (TE, LG, AS), bacterial community analysis (LC, MS, CD, CK, AK), writing of manuscript and preparation of tables, figures and artwork (all authors).

Financing

Authors contributing to this work were partly financially supported by *Microbes-in-Water* GmbH (TE), *bNovate Technologies* SA (TE, SZ, VH, AG, LG, AS). BactoSense flow cytometers used in this study were made available by bNovate Technologies SA and the Interuniversity Cooperation Centre Water & Health. The study was partly financed by Eurostars project MultiSense Aqua (FFG project nr. 871625 and nr. 871615).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121109.

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Thomas Egli reports financial support, article publishing charges, and equipment, drugs, or supplies were provided by bNovate Technologies SA. Thomas Egli reports financial support, administrative support, and article publishing charges were provided by Microbes-in-Water GmbH. Thomas Egli reports a relationship with bNovate Technologies SA that includes: equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.