1 **Identifying the underlying causes of biological instability in a full-scale drinking water supply**
2 **system**
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Abstract

Changes in bacterial concentration and composition in drinking water during distribution are often attributed to biological (in)stability. Here we assessed temporal biological stability in a full-scale distribution network (DN) supplied with different types of source water: treated and chlorinated surface water and chlorinated groundwater produced at three water treatment plants (WTP). Monitoring was performed weekly during 12 months in two locations in the DN. Flow cytometric total and intact cell concentration (ICC) measurements showed considerable seasonal fluctuations, which were different for two locations. ICC varied between 0.1 – 3.75 × 10^5 cells mL^{-1} and 0.69 – 4.37 × 10^5 cells mL^{-1} at two locations respectively, with ICC increases attributed to temperature-dependent bacterial growth during distribution. Chlorinated water from the different WTP was further analysed with a modified growth potential method, identifying primary and secondary growth limiting compounds. It was observed that bacterial growth in the surface water sample after chlorination was primarily inhibited by phosphorus limitation and secondly by organic carbon limitation, while carbon was limiting in the chlorinated groundwater samples. However, the ratio of available nutrients changed during distribution, and together with disinfection residual decay, this resulted in higher bacterial growth potential detected in the DN than at the WTP. In this study, bacterial growth was found to be higher (i) at higher water temperatures, (ii) in samples with lower chlorine residuals and (iii) in samples with less nutrient (carbon, phosphorus, nitrogen, iron) limitation, while this was significantly different between the samples of different origin. Thus drinking water microbiological quality and biological stability could change during different seasons, and the extent of these changes depends on water temperature, the water source and treatment. Furthermore, differences in primary growth limiting nutrients in different water sources could contribute to biological instability in the network, where mixing occurs.

Keywords: biological stability, drinking water distribution system, flow cytometry, nutrients, drinking water monitoring

51 **1. Introduction**
52

53 Recent definitions of biologically stable water imply no changes in bacterial characteristics in the
54 distribution network (DN) (Lautenschlager et al., 2013; Prest et al., 2016b), while the first definition
55 was less strict and implied that biologically stable water does not support the growth of
56 microorganisms to a significant extent (Rittmann and Snoeyink, 1984). Although fluctuations in
57 bacterial concentrations do not necessarily indicate pathogen growth, unpredictable/unknown
58 changes in microbiological water quality are undesirable. For instance, high bacterial concentrations
59 might increase health risk, and/or cause problems with drinking water discoloration and corrosion
60 (van der Kooij, 2000). Microbiological water quality can deteriorate during distribution as the result
61 of external contamination and microbial growth. For example, increase of bacterial numbers during
62 distribution could be caused by inadequate water treatment including insufficient disinfectant
63 residuals, changes in water supply operation (Kumpel and Nelson, 2016) and water consumption
64 (Nescerecka et al., 2014), seasonal fluctuations (Prest et al., 2016c; van der Wielen and van der
65 Kooij, 2010), influence of mixing zones and different water sources (Niquette et al., 2001). Long-
66 term changes in bacterial concentrations are mostly attributed to seasonal fluctuations, which are at
67 least partially caused by varying temperatures and resultant changes in bacterial abundance and
68 activity (Prest et al., 2016c; van der Wielen and van der Kooij, 2010). In contrast, short-term instability
69 is normally caused by hydraulic disturbances due to changes in water consumption, which varies
70 depending on time of the day, day of the week, and the purpose of the building (AWWA, 2002;
71 Besmer et al., 2014; Besmer and Hammes, 2016; Nescerecka et al., 2014). Therefore, biological
72 stability should be considered on various time scales and specific investigations are needed to
73 understand the root causes of instability.
74

75 Many previous studies investigated biological stability and the factors influencing bacterial growth in
76 the DN (Pinto et al., 2014; Prest et al., 2016b; van der Kooij, 2000; van der Wielen and van der Kooij,
77 2010; Volk and LeChevallier, 1999). Organic carbon (C), specifically biodegradable dissolved
78 organic carbon (BDOC) and assimilable organic carbon (AOC), is considered to be the primary factor
79 that promotes or limits growth of heterotrophic bacteria in drinking water (LeChevallier et al., 1991;

80 Servais et al., 1989; van der Kooij et al., 1982). However, studies rarely consider inorganic nutrient
81 limitation, or the potential for multiple limitations in the same sample. For example, inorganic nutrient
82 limitation were shown to be essential for biological stability in regions with high concentrations of
83 organic C (Lehtola et al., 1999; Miettinen et al., 1997; Sathasivan et al., 1997). Moreover, although
84 the methods for determination of AOC and microbially available phosphorus (P) already exist, they
85 are nutrient specific, typically used in isolation, and do not allow direct comparison of their influence
86 on biological stability. Thus, a universal approach for the quantification of various microbially
87 available nutrients is necessary.
88
89 A number of new methods to quantify and characterize bacterial biomass allowed to obtain more
90 accurate and detailed information about biological instability *in situ*, such as biofilm formation rate
91 (BFR) (van der Wielen and van der Kooij, 2010), adenosine triphosphate (ATP) measurements (van
92 der Wielen and van der Kooij, 2010; Vital et al., 2012), 16S rRNA gene pyrosequencing (El-
93 Chakhtoura et al., 2015; Pinto et al., 2012), Illumnia MiSeq 16S rRNA gene sequencing (Liu et al.,
94 2016, p. 201), flow cytometric (FCM) total cell concentration (TCC) (Hammes et al., 2010; Prest et
95 al., 2013) and intact cell concentration (ICC) measurements (Lautenschlager et al., 2013;
96 Nescerecka et al., 2014). Nevertheless, available data on temporal bacterial fluctuations and
97 especially long-term monitoring, which enable understanding of the influence of seasonal
98 fluctuations and different water sources on temporal biological stability in a DN, is limited and
99 contradictive. While large data sets obtained with cultivation-based techniques showed clear
100 seasonal/temperature dependence on bacterial growth (Francisque et al., 2009; LeChevallier et al.,
101 1996), ATP showed seasonal fluctuations at only two out of six investigated water treatment plants
102 (van der Wielen and van der Kooij, 2010). Recently, a two-year study of Prest and colleagues (Prest
103 et al., 2016c) demonstrated only minor bacterial growth in the DN despite the significant seasonal
104 fluctuations of temperature and bacterial counts in the treatment plant effluent. When investigating
105 different water sources, especially seasonal changes showed contradicting observations.. For
106 instance, van der Wielen and van der Kooij (2010) showed that anoxic groundwater was the least
107 biologically stable and was strongly influenced by seasons, but another study demonstrated that
108 bacterial growth was the highest, when distributed water was originated from chlorinated surface

109 water or mixed and water temperature was above 15 °C (Niquette et al., 2001). Spatial and short-
110 term temporal (24 h) biological instability of a chlorinated drinking water distribution network were
111 previously described, but the underlining reasons were not identified (Nescerecka et al., 2014). In
112 that study, some evidence was found of instability in mixing zones where treated surface water and
113 treated groundwater meet. However, the time period of that study was limited to two weeks (in
114 summer) (Nescerecka et al., 2014), which did not allow the observation of seasonal fluctuations.
115 Investigation of seasonal fluctuations and growth-limiting nutrients in various locations in the DN,
116 supplied from groundwater and surface water, is particularly important to understand an influence of
117 different water sources on biological stability in the same DN.
118
119 In the present study we assessed microbial fluctuations in a complex full-scale distribution system
120 supplied from three different water sources during 12 months at a weekly frequency using FCM TCC
121 and ICC. Moreover, we used a modified approach to measure and identify the reasons of biological
122 (in)stability in the treated and distributed water, based on the assessment of a range of growth-
123 limiting/promoting compounds, namely, C, P, nitrogen (N) and iron (Fe). The specific goals were to
124 (1) investigate temporal biological (in)stability on a seasonal (long-term) scale, and (2) identify the
125 growth limiting/promoting factors in the specific system.
126

127 **2. Materials and Methods**

128

129 **2.1. Study site**

130 Sampling was performed in the full-scale distribution network of Riga (Latvia) with a total length of
131 about 1400 km. The network mainly consists of cast iron (80 %) and unlined iron (15 %) pipes. The
132 city is supplied with drinking water from six water treatment plants (WTP) produced from both surface
133 and groundwater (150 000 m^3 d^{-1}). Only the three major WTPs, which are continuously operated,
134 were included in the sampling campaign. These WTPs include full-scale treatment of surface water
135 and chlorination of artificially recharged and natural groundwater (Figure 1), referred as WTP 1, WTP
136 2 and WTP 3 further in the text, accordingly. DN samples were taken from taps at different locations
137 in the city network.

138

139 **2.2. Long-term monitoring in drinking water distribution network**

140 Long-term monitoring was performed at two locations in the DN with different water ages and
141 different source water origins ((1) and (2) at Figure 1, DN1 and DN2 further in the text). For each
142 location, the source water origin and drinking water age were computed according to the Riga DN
143 hydraulic model on the Bentley WaterGEMS® platform. The DN1 was a household tap in the area
144 where water originated from surface water (80 – 100 % of surface water according to modelling data).
145 Drinking water at the DN2, which is a tap in a public building on the university campus, was defined
146 as mixed water, since modelling data suggests 62 – 97 % groundwater during the sampling period
147 (data not shown). Sampling was performed every week during one year (April 2015 – April 2016).
148 The taps were flushed (10 min with average flow rate 0.21 m^3 h^{-1}) before sampling to minimize the
149 influence of stagnation and local building plumbing. Each sample was measured for ICC and TCC
150 in triplicate. Additionally, the water temperature was measured in the DN2. Temperature and total
151 chlorine data from WTPs were provided by "Rīgas Ūdens" water supply provider (Figure S1.1, S1.2).

152

153

154 **2.3. Characterization of biological stability and identifying growth limitation compounds in**
155 **drinking water**

156 Samples were collected at three WTPs before and after the final chlorination step (see residual
157 chlorine concentrations in Table 1 and Figure S1.2), and from the two household taps, attributed to
158 different water sources and treatments: treated surface water DN1 ((1), Figure 1) and chlorinated
159 groundwater DN3 ((3), Figure 1). Natural and artificially recharged groundwater were not specified
160 as separate water sources for DN samples and were defined as "groundwater" when compared to
161 DN3 due to close proximity of WTP 2 and WTP 3 and the inability to determine the exact source
162 during distribution. Additionally, raw water samples were collected at the surface water WTP.
163 Sampling was performed twice: in August 2015 (further in the text as "summer"), and in January
164 2016 ("winter"). The winter sampling campaign was conducted during a period of increased
165 chlorination, which was a short-term (3-5 days) regular procedure aimed to disinfect the water
166 distribution pipes (Figure S1.2). Temperature, free and total chlorine measurements were conducted
167 on site at the time of sampling (Table 1), while samples for FCM and growth potential were collected
168 in AOC-free glass bottles and were analyzed within 8 h of sampling. A modified growth potential
169 method (Section 2.4) was applied to the samples from the summer sampling campaign to identify
170 growth-limiting compounds.

171

172 **Table 1.** Free and total chlorine data, and temperature measurements at three WTPs effluents,
173 measured in summer (normal operation), and winter (increased chlorination) during our sampling
174 campaign (1-year chlorine data demonstrated on Figure S1.2, supplementary information).

	August			January (increased chlorination)		
	Cl_2 Free, mg L^{-1}	Cl_2 Total, mg L^{-1}	t, °C	Cl_2 Free, mg L^{-1}	Cl_2 Total, mg L^{-1}	t, °C
WTP 1	0.03	0.21	20.4	0.73	1.05	1.3
WTP 2	0.06	0.36	8.9	0.93	1.31	8.1
WTP 3	0.11	0.22	9.1	0.92	1.17	8.1

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179 **2.4. Modified growth potential method**

180 The growth potential method was modified from the method described in a recent study (Prest et al., 2016a). The following stock solutions were prepared and used in the experiment: 10 mM NaS_2O_3 as chlorine quenching solution (1 % v/v final concentration); 1 g-C L^{-1} sodium acetate $C_2H_3NaO_2$ as C-source (1 mg-C L^{-1} final concentration); 1.28 g L^{-1} $Na_2HPO_4 * 2H_2O$, 0.3 g L^{-1} KH_2PO_4 as P-source (0.1 % v/v final concentration); 1.77 g L^{-1} $(NH_4)_2SO_4$ as N-source (0.1 % v/v); acidified trace element solution (8 g L^{-1} $CaCO_3$, 1.15 g L^{-1} $MnCl_2 * 4H_2O$, 0.146 g L^{-1} $CuSO_4 * 5H_2O$, 0.13 g L^{-1} $CoCl_2 * 6H_2O$, 0.4 g L^{-1} ZnO, 0.124 g L^{-1} H_3BO_3, 13.42 g L^{-1} $MgCl_2 * 6H_2O$, 1.04 g L^{-1} $Na_2MoO_4 * 2H_2O$ with 0.64 % HCl) (2 μL 100 mL^{-1} final concentration); 10 mM $FeCl_3$ (10 μL 100 mL^{-1} final concentration) and bottled mineral water (Evian, France) was used as natural bacterial inoculum (10^3 cells mL^{-1} (1 % v/v) final concentration). All samples were prepared in AOC-free glass bottles filled with 100 mL of water and then divided into 3 AOC-free glass vials with 20 mL of sample in each. Direct growth potential was determined as sample incubation without any additives. General nutrient limitation was evaluated by addition of NaS_2O_3 (quenching of disinfectant) and a bacterial inoculum (bottled mineral water). Maximal growth potential was tested by addition of quenching solution, bacterial inoculum, and nutrients in excess relative to 1 mg-C L^{-1} $C_2H_3NaO_2$. Finally, growth limitation by different nutrients was tested similarly to maximum growth without addition of the target nutrient source (Table 2). Samples were incubated at 30 °C with shaking (150 rpm) for 72 h. FCM-TCC measurements were performed before and after incubation.

198

199 **Table 2.** Combinations of additives used for the modified growth potential method.

	NaS_2O_3	inoculum	acetate	phosphates	nitrogen	iron
Direct growth	-	-	-	-	-	-
Quenching	+	+	-	-	-	-
C-limitation	+	+	-	+	+	+
P-limitation	+	+	+	-	+	+
N-limitation	+	+	+	+	-	+
Fe-limitation	+	+	+	+	+	-
Max growth	+	+	+	+	+	+

200

201

202

203 **2.5. Fluorescent staining and FCM of water samples**

204 FCM analysis was based on the methods described previously (Berney et al., 2008; Hammes et al.,
205 2008; Nescerecka et al., 2016; Prest et al., 2013). For TCC staining, a working solution of 100x
206 diluted SYBR® Green I (SG) was used. 1 mL of the sample was stained with SG working solution at
207 10 μL mL^{-1} and incubated 10 minutes at 35 °C before analysis. For ICC staining, propidium iodide
208 (PI; 30 mM) was mixed with the SYBR® Green I working solution (SGPI) to a final PI concentration
209 of 0.6 mM. 1 mL of the sample was stained with SGPI at 10 μL mL^{-1} and incubated 15 minutes at
210 35 °C before analysis. Prior to FCM analysis, the water samples were diluted (10 % v/v) with 0.22
211 μm filtered commercially available bottled water (Evian, France). FCM measurements were
212 performed on the CyFlow® SL, equipped with a blue 25 mW solid state laser emitting light at a fixed
213 wavelength of 488 nm. Green fluorescence was collected at 520 ± 10 nm, red fluorescence above
214 630 nm, and high angle sideward scatter (SSC) at 488 nm. The trigger/threshold was set on the
215 green fluorescence channel and data were acquired on two-parameter density plots, while no
216 compensation was used. All data were processed with the FCM propriety software, and electronic
217 gating was used to separate positive signals from instrument and water sample background (Prest
218 et al., 2013).
219
220
221 **2.6. Chlorine concentration determination**
222 Free and total chlorine was measured using commercially available DPD reagent powder pillows
223 (Hach®) and a portable photometer (DR 890, Hach®). Analysis was done as described in the user's
224 manual.
225
226
227 **2.7. Statistical analysis**
228 Statistical analysis was made using MS Excel ANOVA Single factor data analysis tool.
229

3. Results and Discussion

Three WTPs, supplied with different types of water, and three DN samples were analyzed in order to assess biological stability and bacterial dynamics during different seasons in a chlorinated drinking water supply system. Grab samples were taken at each of the three WTPs before and after chlorination and at two DN points (DN1 and DN3), corresponding to the surface water WTP and groundwater WTPs respectively, in winter and summer and analyzed with FCM TCC and ICC (Section 3.1). Tap water from two DN locations – DN1 and DN2 – was analyzed with FCM TCC and ICC weekly during one year (Section 3.2). A modified growth potential assay was applied to the samples from the WTP effluents and DN1 and DN3 samples (Sections 3.3 and 3.4).

3.1. Biological stability of groundwater and surface water during different seasons

Drinking water that originated from surface water was not biologically stable. An increase in cell concentrations was observed in the DN samples, when compared to the surface water WTP (WTP 1) effluent, obtained on the same date (Figure 2A, 2B). For example, ICC increased from $6.27 \pm 0.48 \times 10^4$ cells mL^{-1} after chlorination to $2.92 \pm 0.05 \times 10^5$ cells mL^{-1} in the DN1 in summer and from $1.11 \pm 0.15 \times 10^3$ to $9.95 \pm 1.15 \times 10^3$ cells mL^{-1} in winter. The lower absolute increase of ICC in winter could be explained by less bacterial growth due to the lower temperatures (20.4 °C in summer vs. 1.3 °C in winter) (Table 1, LeChevallier et al., 1996) and higher chlorine residuals during the winter sampling period. Although ICC increase in winter remained in the same order of magnitude and was relatively low, it represents a presence of bacteria in drinking water that either survived chlorination and were able to growth in low temperature, or were detatched biofilm bacteria also resistant to disinfectant. Intensified chlorination was applied during winter, which resulted in 0.73 ± 0.06 mg L^{-1} of free chlorine (1.05 mg L^{-1} as total chlorine) at the WTP 1 and 0.23 ± 0.09 mg L^{-1} of free chlorine in the DN1. In comparison, free chlorine was 0.03 ± 0.006 mg L^{-1} (0.21 mg L^{-1} as total chlorine) after chlorination in summer, which is a normal chlorination practice in the surface water WTP. According to a recent study and World Health Organization guidelines, 0.5 mg L^{-1} of free chlorine is a threshold for effective disinfection, and 0.2 mg L^{-1} at point of the delivery in low-risk systems (Gillespie et al.,

259 2014; WHO, 2008). Moreover, higher bacterial concentrations were observed when free chlorine
260 was less than 0.1 mg L^{-1} in our previous study (Nescerecka et al., 2014), thus bacterial growth in
261 summer was expected.
262
263 The microbiological quality of drinking water originating from chlorinated groundwater also changed
264 during distribution. The DN3 samples could have originated from both/either groundwater WTPs due
265 to the close proximity of the latter, thus the DN3 values cannot be compared to the individual raw
266 water samples directly (Figure 2C-2F). However, ICC values were significantly higher in the network
267 in comparison to both groundwater WTPs effluent values (Figure 2).
268
269 Seasonal differences in cell concentrations were substantial in the surface water after chlorination.
270 Both TCC and ICC were at least 1 log lower in winter than in summer (Figure 2A, 2B), and since the
271 chlorinated system was investigated, differences between ICC were the most distinctive. ICC before
272 chlorination were $4.44 \pm 0.1 \times 10^5$ cells mL^{-1} in summer, and $3.03 \pm 0.17 \times 10^3$ cells mL^{-1} in winter
273 (1 % of ICC in summer). ICC after chlorination in summer were $6.27 \pm 0.48 \times 10^4$ cells mL^{-1} and 1.11
274 $\pm$ 0.15 $\times 10^3$ cells mL^{-1} in winter (2 % of ICC in summer) (Figure 2). Seasonal differences were
275 significant for raw surface water quality too. TCC and ICC of raw surface water in summer were 3.78
276 $\pm 0.12 \times 10^6$ and $2.82 \pm 0.1 \times 10^6$ cells mL^{-1} respectively, and $1.28 \pm 0.01 \times 10^6$ and $1.08 \pm 0.03 \times$
277 10^6 cells mL^{-1} in winter, which was 34 % of TCC and 38 % of ICC in raw water samples in summer
278 (supplementary, Figure S2). However, it is unlikely that changes in raw water bacterial
279 concentrations directly influenced the bacterial concentrations in the WTP 1 effluent. The biofiltration
280 step in the WTP 1 is preceded by ozonation, which is known to inactivate/destroy the majority of
281 bacteria (Ramseier et al., 2011; Vital et al., 2012; von Gunten, 2003). Hence, bacteria detected
282 before chlorination most likely originated from the biofilters (Figure 1) (Lautenschlager et al., 2014;
283 Prest et al., 2016c; Vital et al., 2012). Low bacterial numbers in the WTP 1 during winter suggest
284 that the low temperatures inhibited bacterial growth during biofiltration at the surface water WTP
285 (Figure 2, Figure S2). However, there is no clear consensus about the influence of water temperature
286 on the amount of biomass in the biofilters. While some studies showed decreased amount of
287 biomass on the filter media at low temperatures (Emelko et al., 2006; Moll et al., 1999), others did

288 not observe differences in biomass levels at different temperatures (Fonseca et al., 2001; Servais et
289 al., 1992). Hypothetically, the availability of certain nutrients could also have a seasonal pattern,
290 where lack of nutrients could result in lower bacterial concentrations. However, this is not likely to be
291 the case in the present study, since our results show higher amount of microbially available C and
292 P in winter (Figure 3). These observations are similar to those reported recently by Prest and
293 colleagues (Prest et al., 2016c), which also showed lower suspended bacterial concentrations in
294 biofilter effluent at low seasonal temperatures, even in the presence of AOC. Moreover, several
295 studies showed decreased organic C and inorganic compounds removal in biofilters at colder
296 temperatures (Fonseca et al., 2001; Hoyland et al., 2014; Moll et al., 1999; Servais et al., 1992).
297 Thus we argue that the seasonal differences in cell concentrations was the result of a temperature
298 effect on bacterial growth.
299
300 The difference in bacterial concentration between summer and winter was smaller in groundwater
301 samples than in surface water samples. TCC in artificially recharged groundwater before chlorination
302 was not significantly different between summer and winter ($P > 0.05$), and was $4.83 \pm 0.15 \times 10^5$
303 cells mL^{-1} in summer and $4.77 \pm 0.18 \times 10^5$ cells mL^{-1} in winter (99 % of TCC in summer) (Figure 2C,
304 2D). The seasonal difference was significant ($P < 0.05$) in natural groundwater, where TCC values
305 were $2.36 \pm 0.06 \times 10^5$ cells mL^{-1} in summer and 1.81×10^5 cells mL^{-1} in winter (77 % of TCC in
306 summer). ICC were different in both types of groundwater. ICC in artificially recharged groundwater
307 before chlorination were $3.89 \pm 0.01 \times 10^5$ in summer and $3.23 \pm 0.16 \times 10^5$ in winter (83 % of ICC
308 in summer). In natural groundwater before chlorination ICC were $1.87 \pm 0.08 \times 10^5$ cells mL^{-1} in
309 summer and $1.31 \pm 0.08 \times 10^5$ cells mL^{-1} in winter (70 % of ICC in summer). Although groundwater
310 bacterial composition was not affected by ozonation and biofiltration treatment as in surface water
311 at WTP 1, these striking differences in seasonal fluctuations between groundwater and surface water
312 bacterial concentration are also linked to changes of water temperature, which is one of the main
313 factors influencing bacterial abundance and activity (Francisque et al., 2009; LeChevallier et al.,
314 1996; Prest et al., 2016c; van der Wielen and van der Kooij, 2010). Surface water was exposed to
315 seasonal changes more than groundwater: at the WTP 1, water temperature followed seasonal trend
316 and ranged between 2 – 22 °C during 12 months (n = 325) (Figure S1.1, supplementary information),

317 and was 20.4 – 21 °C during summer sampling and 0.8 – 1.3 °C at the time of winter sampling
318 campaign. In contrast, groundwater temperature was more constant during the year, e.g. 8.7 –
319 10.6 °C at WTP 2 and WTP 3 in summer and 6.7 – 8.1 °C in winter during our sampling campaign,
320 and 6.8 – 8.2 °C during 1-year weekly monitoring at both WTPs (n = 52 (WTP 2), n = 53 (WTP 3),
321 Figure S1.1, supplementary information).
322
323 Our WTP data suggests that fluctuations of water temperature affect both initial bacterial
324 concentrations and individual water treatment processes. Moreover, the results show that bacterial
325 growth occurred in the DN and it is dependent on the season and the water source.
326
327 **3.2. Temporal fluctuations of bacterial concentrations in the DN**
328
329 Clear seasonal fluctuations in TCC and ICC were observed in the DN1 and DN2 samples over 12
330 months (n = 48) (Figure 4, 5). TCC values of the DN1 samples that originated from WTP 1 varied
331 from 0.67 to 5.87 × 10^5 cells mL^{-1}, with a mean value 2.75 ± 1.41 × 10^5 cells mL^{-1} (n = 48) (Figure 4),
332 and ICC values varied from 0.1 – 3.75 x 10^5 cells mL^{-1}, with a mean value 1.46 ± 0.95 × 10^5 cells
333 mL^{-1} (n = 48). Generally higher bacterial concentrations were observed during summer: all ICC and
334 TCC values that exceeded the average occurred between May to October, following a similar trend
335 as the temperature at WTP 1 (Figure S1.1, supplementary information). A strong correlation between
336 bacterial counts in the DN1 samples and water temperature of the WTP 1 effluent was obtained for
337 TCC (R^2 = 0.82; Figure S3A) and ICC (R^2 = 0.8; Figure S3B) (n = 37). The percentage ICC during
338 the observation period was relatively stable: 50 ± 11 % (Figure S4A) without sharp changes, except
339 a drop due to intensified chlorination (end of January) (Figure S1.2).
340
341 Our data corroborate previous studies, where generally higher bacterial concentrations were
342 observed in warm water in comparison to cold water temperatures (Francisque et al., 2009;
343 LeChevallier et al., 1996; Liu et al., 2013; Prest et al., 2016c; van der Wielen and van der Kooij,
344 2010). A particularly interesting example was demonstrated in the study of Prest and colleagues
345 (2016), where long-term monitoring was performed systematically in the DN with FCM, and a similar-

346 appearing seasonal trend of bacterial concentrations was shown (Prest et al., 2016c). However, in
347 contrast to the present study, these fluctuations were predominantly occurring at the WTP (during
348 biofiltration), and only minor additional growth was observed in the DN. In the current study, TCC
349 and especially ICC differences between WTP effluent and DN samples indicate that the dominant
350 bacterial growth occurred in the distribution system (Figure 2), corresponding with the data from the
351 earlier study, where increase of ICC was observed in the same DN in the distal areas from the WTPs
352 (Nescerecka et al., 2014). Moreover, the data demonstrated in Figure 2 also show that ICC increased
353 more dramatically than TCC values in the chlorinated DN. Therefore, measuring ICC for bacterial
354 growth characterization is particularly important when evaluating biological stability after disinfection.
355
356 TCC and ICC values of the mixed DN2 samples were different from the surface water DN1 samples
357 and showed less seasonal dependency. TCC ranged from 1.98 – 6.76 × 10^5 cells mL^{-1} (mean = 4.41
358 ± 1.36 × 10^5 cells mL^{-1} (n = 48)) (Figure 5), and ICC values varied from 0.44 – 4.37 × 10^5 cells mL^{-1}
359 (mean = 2.20 ± 1.05 × 10^5 cells mL^{-1}; n = 48), thus both TCC and ICC were significantly different
360 from the DN1 samples ($P < 0.05$). Differences between summer and winter in the DN2 were not as
361 apparent as in the example of DN1 samples. However, clearly lower TCC and ICC values were
362 observed in a period from mid-January until April. Temperature in the DN2 water tap ranged from
363 11.5 to 19.3 °C during the entire observation period, and relatively weak correlations between DN2
364 water temperature and TCC (R^2 = 0.56) (Figure S5A) and ICC (R^2 = 0.49) (Figure S5B) were
365 obtained. Similarly weak correlations were obtained between bacterial concentration in the DN2 and
366 water temperature at the WTPs: water temperature at WTP 1 (surface water) correlated with DN2
367 TCC with R^2 = 0.46 (Figure S6A), and ICC with R^2 = 0.42 (Figure S6B); while temperature at the
368 WTP 2 (groundwater) correlated with TCC and ICC with R^2 = 0.51 (Figure S6C) and R^2 = 0.55 (Figure
369 S6D), respectively. The weaker correlation between bacterial counts and water temperature at WTP
370 1 in the mixed DN2 samples in comparison to the surface DN1 samples (Figure S3) indicates that in
371 the DN2 sample, changes in bacterial concentrations were less dependent on WTP 1 variables.
372 Microbiological water quality and bacterial growth in the DN2 were more influenced from
373 groundwater sources (60-100 % groundwater) and/or were affected by longer water retention time
374 (water age) than DN1 samples (0-20 % groundwater).. For example, according to the modelling data,

375 the water age of surface water in the mixed DN2 sample was 42 – 68 h during sampling time
376 (groundwater water age was not available), in contrast to the DN1 samples, where it was ca. 32 –
377 50 h. Apparently, longer water age in the DN2, and, therefore, increase of water temperature,
378 chlorine decay, possible "mixing" of different water and bacterial growth, could explain more different
379 drinking water quality parameters from WTP 1 than in the DN1 with shorter water age.
380
381 Moreover, the percentage ICC pattern in the DN2 was different from the DN1. The percentage ICC
382 was less stable in the DN2 samples with a mean value 49 ± 15 %, ranging from 21 – 84 % (Figure
383 S4B). Noticeable peaks in the percentage ICC were observed during mid-July to mid-August, and
384 mid-October to mid-December. We do not have evidence of what could be the exact reason of this
385 phenomena, but we believe that the increased ICC were the result of bacterial growth in the network,
386 and the difference in the percentage ICC depended on hydraulic conditions and water age. For
387 example, a decrease of water consumption could lead to water stagnation, and changing water
388 demand could also lead to a change of the sourse water and source water "mixing", which could
389 have different ICC percentages and/or enhance bacterial growth (Section 3.3.). An ICC percentage
390 increase could also result from bacteria detachment from biofilms due to increased shear stress, and
391 intensified afterward bacterial growth, resulting from increased mass transfer of the nutrients (Horn
392 et al., 2003; Lehtola et al., 2006; Tsai, 2005). Moreover, peaking ICC were observed on a daily basis
393 in the same DN before (Nescerecka et al., 2014), therefore the percentage ICC peaks in the DN2
394 could be related to the shifts in TCC and ICC daily variations.
395
396 Although only limited data of the detailed long-term monitoring in the distribution network is available,
397 there is evidence that water biological parameters are affected by the season, and in most cases
398 these changes are inevitable (Francisque et al., 2009; LeChevallier et al., 1996; Niquette et al., 2001;
399 Prest et al., 2016c).
400
401 **3.3. Growth-limiting nutrients in the WTP samples from different water sources**
402

403 Surface water and groundwater after chlorination have different growth-promoting/growth-limiting
404 properties. In this study we employed and expanded a growth potential assay to assess for multiple
405 limitations (Table 2, (Prest et al., 2016a)). Bacterial growth was not detected in WTP 2 and WTP 3
406 after chlorination following direct incubation, while growth in the WTP 1 effluent was $6.84 \pm 5.54 \times$
407 10^4 cells mL^{-1} (Figure 6). If it is assumed that C is the limiting nutrient, this growth is equivalent to
408 6.8 µg AOC L^{-1} in WTP 1 effluent (Vital et al., 2012). This value meets the requirements for biological
409 stability of chlorinated drinking water (50 - 100 µg AOC L^{-1}), proposed by various authors
410 (LeChevallier et al., 1991; Polanska et al., 2005). While bacterial growth in the samples with addition
411 of chlorine quenching solution ($Na_2S_2O_3$) and a bacterial inoculum (“Direct + Q + in” sample) was not
412 significantly different from direct incubation growth in the WTP 1 effluent sample, it resulted in large
413 ($4.38 – 7.12 \times 10^5$ cells mL^{-1}) increases in WTP 2 and 3 effluent samples. The absence of more
414 growth in the WTP 1 effluent sample after quenching clearly indicates a nutrient limitation. The
415 increased growth in the WTP 2 and WTP 3 samples indicates growth limitation by residual chlorine
416 in the initial sample (direct incubation results). However, it should be noted that the addition of
417 $Na_2S_2O_3$ as a chlorine quenching solution could serve as a nutrient for certain bacterial species
418 (Chien et al., 2007; Maclean et al., 1996).
419
420 P was the primary growth-limiting nutrient in the treated and chlorinated surface water sample (WTP
421 1). When all other nutrients were in excess, the available P in the water allowed $2.07 \pm 1.75 \times 10^5$
422 cells mL^{-1} net growth, which was not statistically different from the result of the quenched sample
423 ($1.83 \pm 1.78 \times 10^5$ cells mL^{-1}, $P > 0.05$) without additional nutrients (Figure 6A). C was the second
424 growth-limiting nutrient. However, the water contained sufficient organic C to promote 10-fold higher
425 net growth than P ($2.06 \pm 0.22 \times 10^6$ cells mL^{-1}, equivalent to 206 µg AOC L^{-1}) when all other
426 compounds were in excess. P was considered as a main limiting nutrient in water in various countries,
427 including Northern European countries and Japan, where high organic content is typical for natural
428 water (Miettinen et al., 1996, 1997; Sathasivan et al., 1997).
429
430 In contrast, organic C was the primary growth-limiting nutrient in the chlorinated groundwater
431 samples. The amount of C in the WTP 2 effluent could promote $9.42 \pm 0.47 \times 10^5$ cells mL^{-1} net

432 growth (ca. 94 µg AOC L^{-1}), and the net growth in WTP 3 without addition of C was 7.12 ± 0.37 x 10^5
433 cells mL^{-1} (ca. 71 µg AOC L^{-1}), which was not statistically different from the quenched sample (6.4 ±
434 0.91 × 10^5 cells mL^{-1}, $P > 0.05$). Bacterial growth, obtained in conditions without additional P, was
435 not statistically different from the samples with all nutrients added, which shows that P was not a
436 growth-limiting nutrient in either groundwater. This therefore implies that P is naturally present in the
437 groundwater in concentrations sufficient for bacterial growth. Although total organic carbon (TOC)
438 and P were not measured in the present study, a previous study showed a relatively high TOC
439 content in the groundwater: 3 and 9 mg L^{-1} at WTP 2 and WTP 3, respectively, while it was 6 mg L^{-1}
440 at WTP 1 (Nescerecka et al., 2014). This indicates that the AOC fraction represents less than 3.3 %
441 of TOC, and they do not correlate. Therefore, we argue that the measurements of biologically
442 available nutrients, especially small concentrations specific for drinking water, are better indicators
443 for biological stability than measurements of their total concentrations.
444
445 This data shows that water from different origins, distributed in the same network, has different and
446 potentially complementary growth-limiting nutrients, demonstrating the challenges faced by large
447 water utilities, which use different water sources and treatment for drinking water production. This
448 shows that it is critical to assess not only AOC (LeChevallier et al., 1996; Polanska et al., 2005; van
449 der Kooij, 1992), but rather carefully distinguish between different growth limiting compounds to
450 understand biological instability (Miettinen et al., 1996, 1997, Prest et al., 2016a, 2016b).
451
452 **3.4. Growth-limiting nutrients in the DN samples**
453
454 Bacterial growth potential in the drinking water changed during distribution. Water, which initially did
455 not promote bacterial growth, was no longer biologically stable after distribution. Bacterial growth
456 was 3.28 ± 0.78 × 10^5 cells mL^{-1} in the DN1 sample (Figure 7A), and 2.48 ± 0.79 × 10^5 cells mL^{-1} in
457 DN3 sample, when estimated as direct incubation growth (Figure 7B). This was higher than the
458 values obtained in the WTPs effluent (Figure 6), which could be partially explained by low chlorine
459 residuals at the WTP 1 (Table 1) and subsequent chlorine decay. However, particularly interesting
460 is biologically unstable DN1 sample, because initially, besides residual chlorine, growth was limited

461 by P at the corresponding WTP 1 ($6.84 \pm 5.54 \times 10^4$ cells mL^{-1} as direct incubation). Therefore, the
462 observed bacterial growth in the DN1 suggests availability of nutrients that were absent in the WTP
463 1 effluent, particularly P, and an increase of AOC (Section 3.3). Moreover, P became the primary
464 growth-limiting nutrient in both the surface water DN1 and the groundwater DN3 sample. P in the
465 DN3 sample could support only $1.19 \pm 0.47 \times 10^6$ cells mL^{-1} growth (10-fold lower than in WTP
466 sample).
467
468 The mechanism responsible for these nutrients shifts are not entirely clear. Decrease of microbially
469 available P during distribution indicates that it was either consumed by bacteria (Nescerecka et al.,
470 2016) or transformed into other forms of P-containing compounds, which are not microbiologically
471 available. Moreover, Rubulis and Juhna (2007) showed increased numbers of heterotrophic bacteria
472 in a closed reactor biofilm a long time after the P concentration decreased in the bulk water, and
473 suggested that this could be explained by an ability of certain types of organisms to accumulate P,
474 similarly to the mechanism used in biological P removal in wastewater (Harold, 1966; Rubulis and
475 Juhna, 2007). Although P remained the growth-limiting nutrient in surface water, it could promote 5-
476 fold higher bacterial growth in the DN1 than directly after treatment: net growth without additional P
477 in the DN1 sample was $9.76 \pm 3.29 \times 10^5$ cells mL^{-1}. There is no clear evidence of what could be the
478 reason of increased biologically available P concentrations. However, several options should be
479 considered. One of the explanations could be a direct mixture of groundwater and surface water in
480 the reservoirs, which are used for compensation of water consumption fluctuations in the city. As
481 was found out before, these water sources have different growth-promoting properties (Section 3.3).
482 Additionally, P could be released from bacteria as a result of chlorination or other stress (Nescerecka
483 et al., 2016). Other studies showed an increase of P in water as a result of cast iron pipes corrosion
484 and its accumulation and release from biofilms (Douterelo et al., 2016; Morton et al., 2005). Although
485 addition of phosphates for corrosion protection and plumbosolvency control was not applied in the
486 studied DN, and therefore is not explaining changes of P in the current study, it is a common practice
487 in various countries (Douterelo et al., 2016; Volk et al., 2000), and P adsorbed to the pipes could
488 potentially become a nutrient source for bacteria. A plausible explanation of increased P
489 concentrations could be P accumulation in the biofilms from initially P-unlimited groundwater, and

490 its subsequent release. For example, as reported elsewhere, bacterial known for phosphate
491 accumulation such as *Aquabacterium commune* and *Acinetobacter* species were found in drinking
492 water biofilms, where the latter were specifically attributed to groundwater (Kalmbach et al., 2000;
493 Kelly et al., 2014).
494
495 While the AOC difference between the surface water samples before and after distribution was not
496 significantly different ($P > 0.05$), changes in the calculated AOC concentration were observed in the
497 DN3 sample, where it promoted $3.19 \pm 0.18 \times 10^6$ cells mL^{-1} or 3-fold higher growth than in WTP
498 sample. Various hypotheses concerning increase of AOC concentrations could be considered.
499 Several studies showed that AOC could be released as a result of chlorine oxidation of bacteria and
500 complex organic matter (LeChevallier et al., 1996; Liu et al., 2015; Polanska et al., 2005). Biofilms
501 can play an important role in providing nutrients, and particularly organic C that can be consumed
502 by bacteria. For example, a study on interactions between biofilms and humic substances showed
503 that humics, which normally are not degradable by suspended bacteria, could serve as a sole C
504 and energy source for bacteria in presence of biofilms (Camper, 2004). Moreover, various
505 extracellular enzymes potentially are able to degrade biofilm matrix during starvation (Flemming and
506 Wingender, 2010).
507
508 Obviously, changes in growth-promoting parameters in the full-scale network cannot be explained
509 by one single mechanism, it is rather a combination of various processes, occurring due to interaction
510 between bulk water, biofilms, disinfection residuals and pipe materials. However, we also assume
511 that transfer of nutrients is likely to occur in the system, which is supplied from water sources with
512 different initial growth-promoting parameters. Thus, the risk of bacterial growth is higher in such DN,
513 and this should be considered during planning, design and chosing treatment technologies of new
514 WTPs, or and optimization of existing water supply systems.
515

516 **3.5 Implications for water utilities**

517

Higher and seasonally fluctuating bacterial concentrations in the DN in comparison to the WTP data indicate that the DN is not biologically stable. This corroborates our previous study, where an increase of bacterial counts in the DN was observed (Nescerecka et al., 2014), which most likely were caused by bacterial growth. Although we did not study whether the water contained any (opportunistic) pathogens, it is clear that uncontrolled bacterial growth implies potential health risk if these bacteria are present in water. The one-year study demonstrates a seasonal tendency of bacterial concentration changes in the DN, and it differs depending on the location and water source. While multiple source waters feeding the same DN is often unavoidable, our data show that it is imperative for operators to develop an evidence-based understanding of how this impacts biological stability and quality in the overall DN. Seasonal fluctuations should be monitored and considered, when defining a baseline for bacterial concentrations in water (Besmer et al., 2016, 2014; Prest et al., 2016c), operating water treatment plants (final disinfection) and while monitoring DN samples: the same bacterial concentrations could be normal during warm season, but it would indicate contamination or unacceptable growth, if measured in winter. Hence, understanding the seasonal tendency and biological stability in a particular system will allow identification of the reasons of bacterial count changes and potential risks, optimize disinfection maintanence and possibly reduce operational costs.

Although our actual data are specific for this particular drinking water system, the presented approach and observations, namely, temperature-dependent bacterial growth in DN, microbial and nutrient dynamics, a role of different water sources and their differences in growth-promoting nutrient content, are essential to know and to use for characterization of each drinking water system. Moreover, the long-term data of every individual system could be collected and integrated into modeling platforms, allowing to track the changes of drinking water quality, determine the water age and detect irregular deviations from the established baseline.

Normally only heterotrophic plate count (HPC), *E. coli* and coliforms (also determined by cultivation methods) are monitored in drinking water systems, while the major part of microbial community remains undetected and uncharacterized. Thus it results in a large gap in knowledge of actual

547 bacterial abundance, communities and their activity in DN (Ingerson-Mahar and Reid, 2012). The
548 conventional biological methods are not able to detect all bacteria in water, as only small percentage
549 of drinking water bacteria can be cultivated (Hoefel et al., 2003; Van Nevel et al., 2017). Ideally,
550 cultivation-independent and/or pathogen-specific methods, such as FCM, 16S rRNA amplicon
551 sequencing, and target-specific qPCR approaches should be used to get reliable data for the DN
552 microbiological quality characterization. For example, FCM was able to determine seasonal changes
553 and different trends between the DN water samples. Although a comparison between HPC and
554 FCM measurements was not made during this study, weak correlations ($R^2 = 0.18$) were observed
555 between HPC and ICC in the samples from exactly the same DN before (Nescerecka et al., 2014).
556 Based on this, and literature (e.g., Hoefel et al., 2003; Liu et al., 2016, p. 201; Nescerecka et al.,
557 2014; Van Nevel et al., 2017), we argue that FCM-ICC is more valuable as overall microbiological
558 process variable to assess changes in drinking water systems than cultivation methods. Thus it
559 represents a potential approach for systematic drinking microbiological water quality monitoring.
560 However, it is evident that utilities would need time to gain experience and establish a sufficient
561 quantity of background data in order to incorporate such new methods into regular water monitoring
562 programs.
563
564 Multi-step treatment as in the WTP 1 and final chlorination with disinfection residuals at all studied
565 WTPs did not prevent increase of bacterial counts in the DN. In turn, bacterial growth in the DN could
566 be possible only with presence of nutrients and low or zero chlorine residuals. Therefore, the solution
567 to improve microbiological water quality in this specific systems is either to increase disinfection
568 residuals in DN or reduce nutrients in water. In both cases, potential risks and costs should be
569 evaluated. Too high chlorine residuals are generally not recommended due to various drawbacks,
570 including taste and odor and formation of potentially harmful disinfection by-products. While nutrient
571 removal can be a suitable option for prevention of bacterial growth, risk analysis should be
572 undertaken to ensure that water is safe and pathogen-free before final chlorination practice can be
573 cancelled. This approach would most likely also entail intensified DN monitoring and maintenance.
574 If biological stability is achieved by removing nutrients from water, it is important to know and to
575 control specific growth-limiting nutrients in water. An interesting finding in our study about growth

576 limitations by different nutrients in different water sources supplying the same DN emphasize the
577 need to test several nutrients to predict potential risk of bacterial growth, to make water treatment
578 more effective and allow to reduce operational costs (Miettinen et al., 1996). This is especially
579 important at the initial stage of WTP design or water supply system planning or reorganizing. For
580 example, water supply from the same type of source water with the same primary growth-limiting
581 compounds would provide more biologically stable water, and nutrient removal would be more
582 effective. For the systems, where the main growth-limiting nutrients are different due to different
583 water sources, and only one source would not satisfy water demand, it would be recommended to
584 reduce both limiting nutrients to avoid bacterial growth risks if water is mixed.
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591

4. Conclusions

- Two locations in the drinking water DN were monitored with flow cytometric TCC and ICC for one year and higher cell counts at higher water temperatures demonstrated clear but different seasonal biological instability at two locations.
- Increase of bacterial concentration in the DN samples in comparison to WTPs indicated bacterial growth in the network. A more distinctive increase of ICC than TCC emphasized an importance of viability assessment for chlorinated DN microbiological water quality characterization.
- A new modified growth potential assay allowed to determine different growth-limiting compounds, and it was demonstrated that these could be different even in different source waters supplying the same DN: P was the primary growth-limiting nutrient in the chlorinated surface water and organic C – in the chlorinated groundwater.
- Biological instability in the DN from initially stable WTPs effluent water was observed. Bacterial growth potential, the amount and composition of available nutrients in water changed during the distribution: in comparison to corresponding WTP effluent, chlorinated surface water had up to five-fold more microbially available P after distribution, and chlorinated groundwater had three-fold more microbially available C after distribution, but ten-fold less P.
- Increased risk of bacterial growth in the DN occurs if DN is supplied with different water sources with different growth-promoting parameters.
- Drinking water microbiological quality and bacterial growth could change during different seasons, and the extent of these changes depends on water temperature, the water source and treatment.

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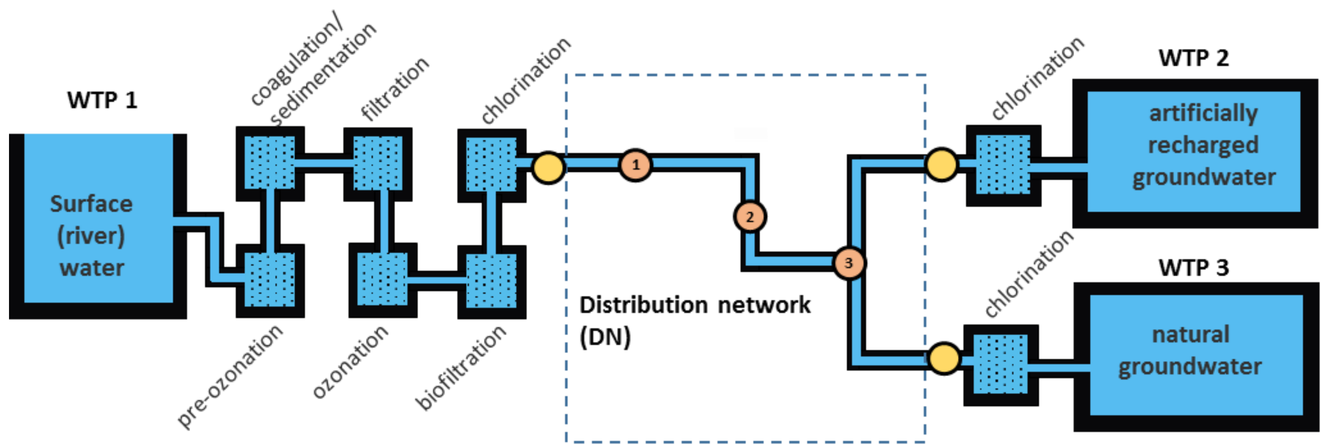

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817 **Figure 1.** A principal scheme of the investigated drinking water supply system. A city was supplied
818 from three main water treatment plants (WTP), which involve full-scale treatment of surface water,
819 and chlorination of natural and artificially recharged groundwater. Samples were taken after
820 chlorination (yellow circles) to investigate growth-promoting nutrients at WTPs. DN samples could
821 be defined as surface water DN1 sample (1), mixed DN2 sample (2) and groundwater DN3 sample
822 (3) according to supplying water sources. Samples DN1 and DN2 were analyzed weekly during one
823 year, while samples DN1 and DN3 were measured to assess limiting nutrients in the DN. Additionally
824 several samples were taken at WTPs (raw water, water before chlorination).

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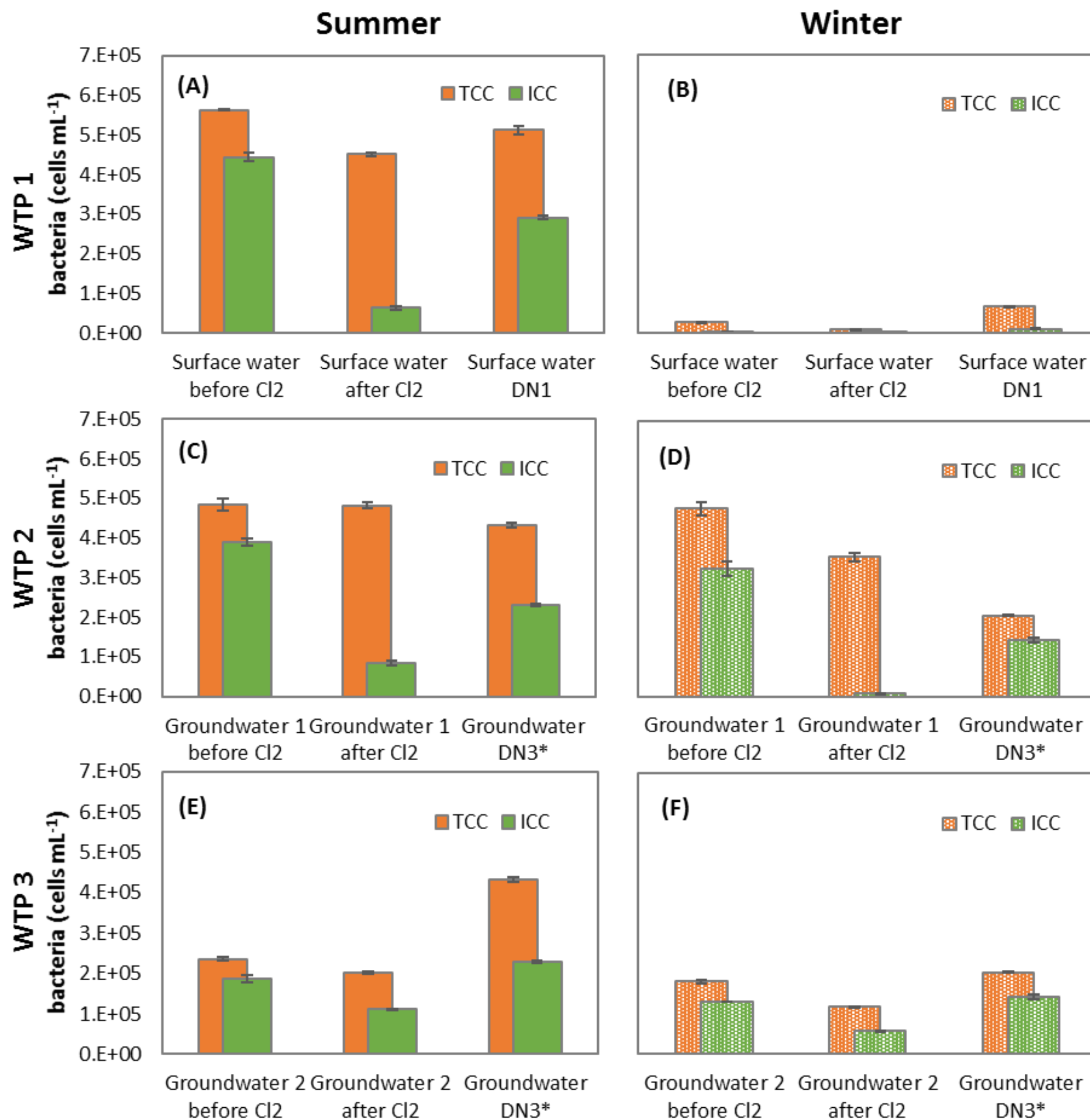

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827 **Figure 2.** ICC and TCC measured with FCM before and after chlorination (Cl2) at the three different WTPs and DN. The samples were measured in summer (A, C, E) and winter (B, D, F). Additionally, increased chlorine dose was applied during the winter sampling campaign. Error bars represent standard deviations calculated from three measurements.

831 **groundwater DN3 sample is the same for both groundwater WTPs due to close location of both WTPs to each other, and thus inability determine the exact origin of the water.*

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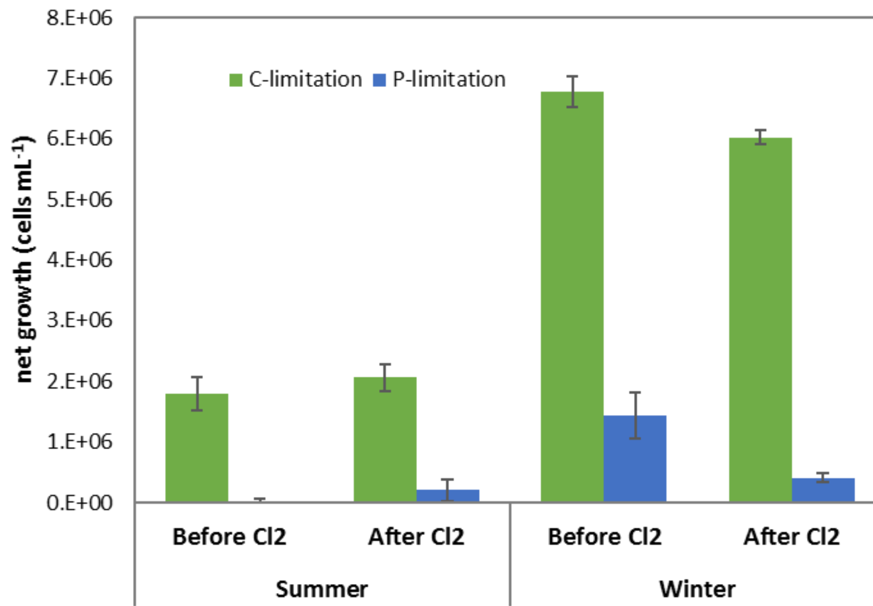

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839 **Figure 3.** Increase in TCC at WTP 1 after 72 h incubation without addition of C (C-limitation) or P
840 (P-limitation) source. Higher growth represents higher amount of the particular nutrient presence in
841 the sample. Error bars represent standard deviations calculated from three separate growth potential
842 tests.
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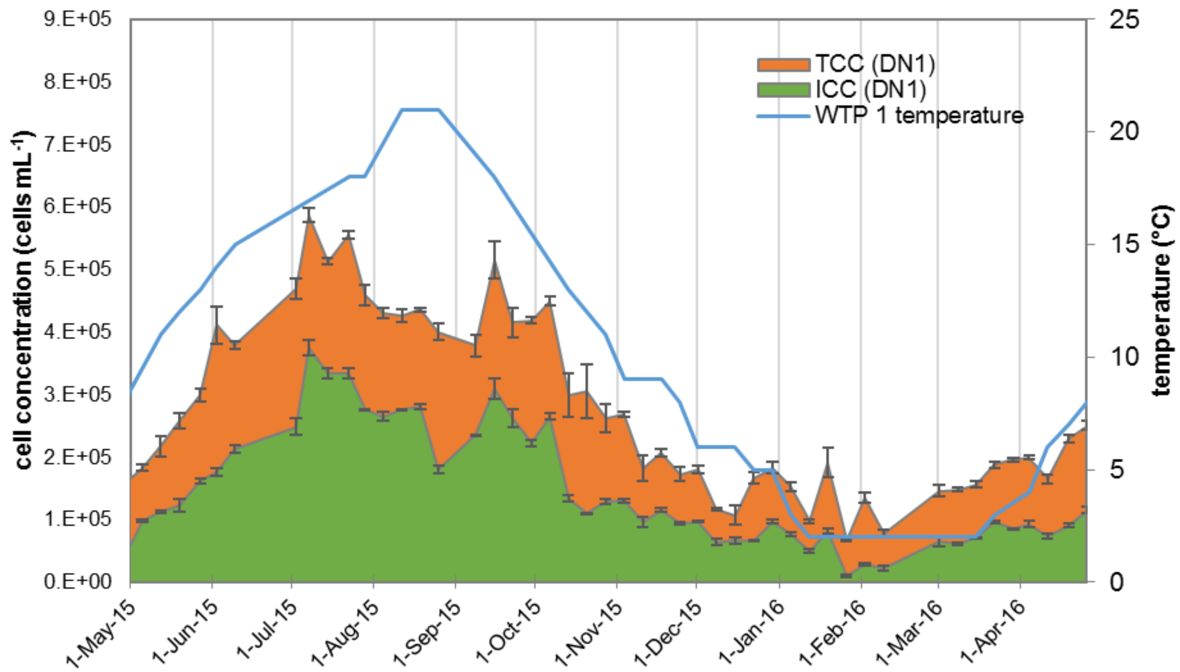

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846 **Figure 4.** Temporal bacterial fluctuations in the DN1, which was supplied with treated surface water,

847 and temperature of finished water at WTP 1 over long-term monitoring (n = 48). Error bars represent

848 standard deviations calculated from three measurements.

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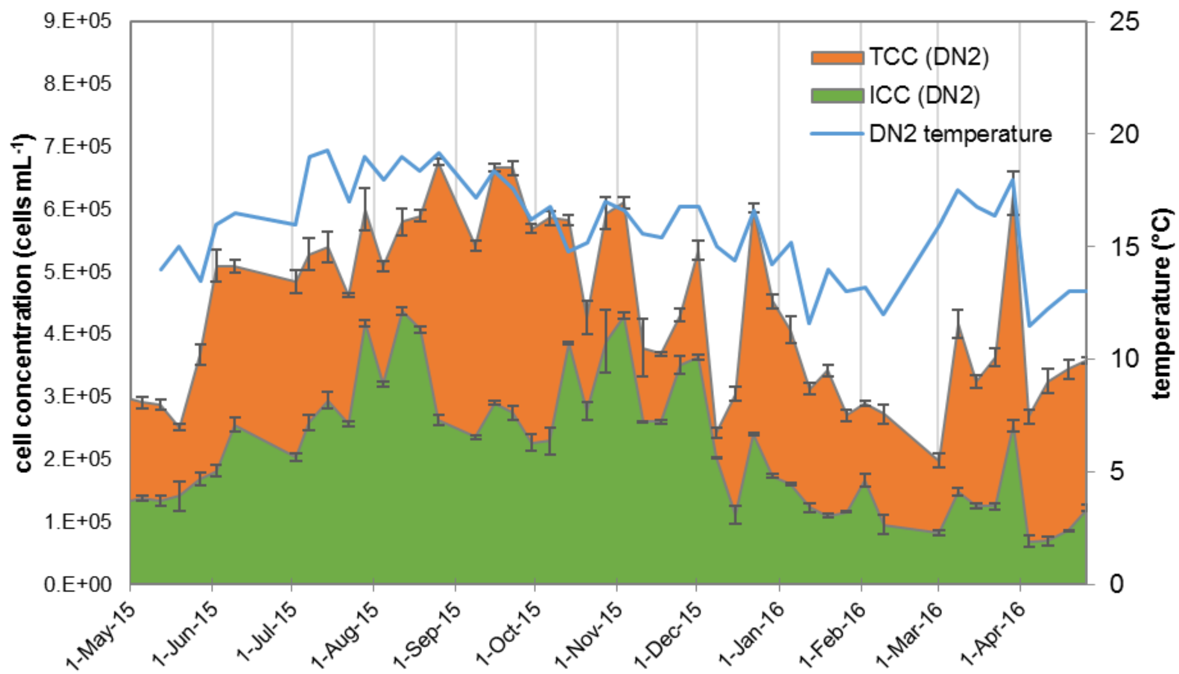

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852 **Figure 5.** Temporal bacterial cell fluctuations and tap water temperature over long-term monitoring
853 of the drinking water DN2 samples, which was originated from mixed water (n = 48). Error bars
854 represent standard deviations calculated from three measurements.

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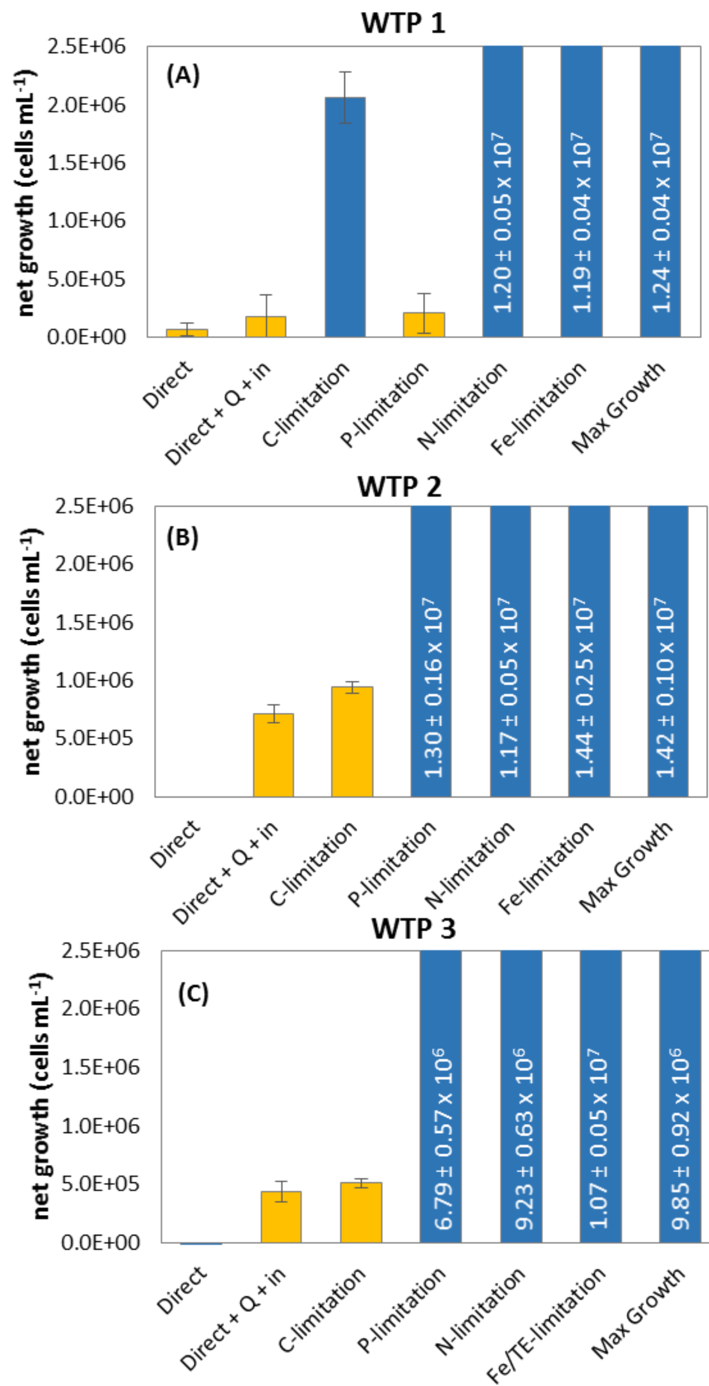

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858 **Figure 6.** Bacterial growth, represented as changes in TCC in the samples from WTP effluent after 72 h incubation. The growth potential approach was tested for effluent water after chlorination at three WTPs supplied with: (A) surface water; (B) artificially recharged groundwater and (C) natural groundwater. The control samples without addition of nutrients (direct and direct + Q +in) and with combination of nutrients, which resulted in the least growth, are marked yellow. Error bars represent standard deviations calculated from three separate growth potential tests.

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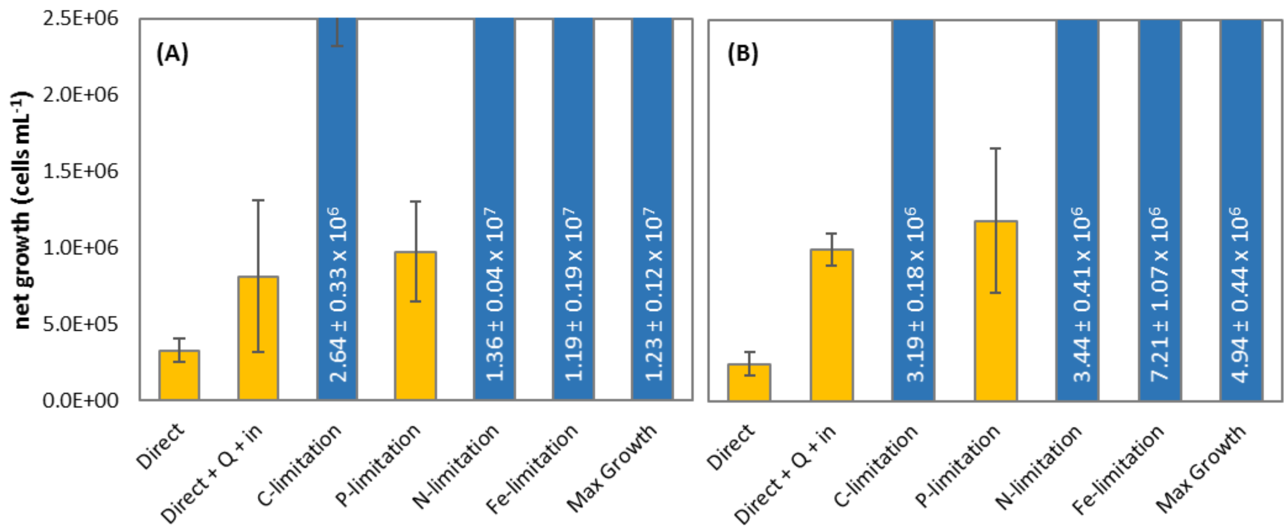

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868 **Figure 7.** Bacterial growth, represented as changes in TCC in the DN samples after 72 h incubation.
869 The new growth potential approach was tested for drinking water DN samples, which are supplied
870 principally from (A) surface water (DN1); (B) groundwater (DN3). The control samples without
871 addition of nutrients (direct and direct + Q +in) and with combination of nutrients, which resulted in
872 the least growth, are marked yellow. Error bars represent standard deviations calculated from three
873 separate growth potential tests.

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876

877 **Table 1.** Free and total chlorine data, and temperature measurements at three WTPs effluents,
878 measured in summer (normal operation), and winter (increased chlorination) during our sampling
879 campaign (1-year chlorine data demonstrated on Figure S1.2, supplementary information).

880

	August			January (increased chlorination)		
	Cl_2 Free, mg L^{-1}	Cl_2 Total, mg L^{-1}	t, °C	Cl_2 Free, mg L^{-1}	Cl_2 Total, mg L^{-1}	t, °C
WTP 1	0.03	0.21	20.4	0.73	1.05	1.3
WTP 2	0.06	0.36	8.9	0.93	1.31	8.1
WTP 3	0.11	0.22	9.1	0.92	1.17	8.1

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882

883 **Table 2.** Combinations of additives, used for modified growth potential method.

	NaS_2O_3	inoculum	acetate	phosphates	nitrogen	iron
Direct growth	-	-	-	-	-	-
Quenching	+	+	-	-	-	-
C-limitation	+	+	-	+	+	+
P-limitation	+	+	+	-	+	+
N-limitation	+	+	+	+	-	+
Fe-limitation	+	+	+	+	+	-
Max growth	+	+	+	+	+	+

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