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Antibiotic resistance in high and low nucleic acid content bacteria

Bachelor thesis

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Abstract

This study focused on the distribution of antibiotic resistance genes (ARGs) in «high nucleic acid (HNA) content bacteria» and «low nucleic acid (LNA) content bacteria» in drinking water treatment plants (DWTPs) with different water sources and treatment steps, and bacterial regrowth within their distribution systems (DWDSs). The aim was to identify potential associations of HNA and LNA content bacteria with antibiotic resistance indicator genes – the class 1 integron-integrase gene *intI1* and the ARG *sul1*. In addition, drinking water facilities with few or no treatment steps were examined to assess whether they could potentially pose a higher risk in case of ARGs or bacterial regrowth and to obtain information on the extent to which flow cytometry (FCM) can contribute to the assessment of microbial risk factors in drinking water.

Water samples from three larger and three smaller DWTPs in Switzerland were filtered on 0.45 μm and 0.2 μm filter membranes to investigate HNA and LNA cells with FCM and additionally, to compare the frequency of *IntI1* and *sul1* in HNA and LNA content bacteria with real-time quantitative polymerase chain reaction (real-time qPCR). Furthermore, real-time qPCR was used for 16S rRNA to monitor bacterial regrowth. FCM proved to be a rapid method for detection and separation of HNA and LNA cells in water samples and will be a good method in further drinking water studies. However, it should be mentioned that FCM will not reveal any effect in UV disinfection, which could give a false impression of the effectiveness of this treatment step.

Interestingly, the ARG *sul1* has never been detected with real-time qPCR in previously treated samples, whereas it could be detected in river water samples with higher HNA content bacteria. Furthermore, the relative abundances compared to 16S rRNA and the proportions in bacteria with HNA and LNA content showed higher frequencies of *intI1* and *sul1* in bacteria with HNA content than in LNA content bacteria. Moreover, considering ARGs - when comparing the proportions of gene copies per cell in HNA and LNA content bacteria - it generally resulted that LNA content bacteria had less than 5% of them. These results showed a potential for the possibility that a minimum genome size for carrying an ARG could exist. However, a potentially higher risk of ARGs was not observed in drinking water systems with fewer or no treatment steps, although it was found that the most effective treatment step to reduce HNA content bacteria is by infiltration of surface water into groundwater. In the future, however, these results would need to be further investigated in other studies aiming for a wider range of ARGs.

Zusammenfassung

Diese Studie konzentrierte sich auf die Verteilung von Antibiotikaresistenzgenen (ARGs) in «Bakterien mit hohem Nukleinsäuregehalt (HNA)» und «Bakterien mit niedrigem Nukleinsäuregehalt (LNA)» in Trinkwasseraufbereitungsanlagen (DWTPs) mit unterschiedlichen Wasserquellen und Behandlungsschritten, sowie auf das Bakterienwachstum innerhalb ihrer Verteilungssysteme (DWDSs). Ziel war es, potenzielle Assoziationen von HNA und LNA Bakterien mit Antibiotikaresistenz-Indikatorgenen (*intI1* und *sul1*) zu identifizieren. Darüber hinaus wurden Trinkwasseranlagen mit wenigen oder keinen Aufbereitungsschritten daraufhin untersucht, ob sie im Falle von ARGs oder Bakterienwachstum ein potenziell höheres Risiko darstellen könnten und inwiefern die Durchflusszytometrie (FCM) zur Beurteilung mikrobieller Risikofaktoren im Trinkwasser beitragen kann.

Wasserproben von drei grösseren und drei kleineren DWTPs in der Schweiz wurden auf 0,45 μm und 0,2 μm Filtermembranen filtriert, um HNA- und LNA-Zellen mit FCM und zusätzlich die Häufigkeit von *IntI1* und *sul1* in HNA und LNA Bakterien mit der quantitativen Echtzeit-Polymerase-Kettenreaktion (real-time qPCR) zu untersuchen. Darüber hinaus wurde die Echtzeit-qPCR für ein Monitoring der 16S rRNA und des Bakterienwachstums verwendet. Die FCM erwies sich als eine schnelle Methode zum Nachweis und zur Trennung von HNA- und LNA-Zellen in Wasserproben und wird eine gute Methode in weiteren Trinkwasserstudien sein. Es sollte jedoch erwähnt werden, dass die FCM keine Wirkung bei der UV-Desinfektion zeigt, was einen falschen Eindruck von der Wirksamkeit dieses Behandlungsschrittes vermitteln könnte.

Interessanterweise wurde das ARG *sul1* nie mit Echtzeit-qPCR in zuvor behandelten Proben nachgewiesen, wohingegen es in Flusswasserproben mit höherem HNA-Gehalt entdeckt werden konnte. Weiter zeigten die relativen Häufigkeiten im Vergleich zu 16S rRNA und die prozentualen Anteile in HNA und LNA höhere Frequenzen von *intI1* und *sul1* in HNA Bakterien. Zudem resultierte in Anbetracht von ARGs – beim Vergleich der Verteilung von Genkopien pro Zelle in HNA und LNA Bakterien – im Allgemeinen, dass Bakterien mit LNA-Gehalt weniger als 5% davon besassen. Diese Ergebnisse zeigten ein Potenzial für die Möglichkeit, dass eine Mindestgenomgröße für das Tragen von ARGs existieren könnte. Ein potenziell höheres Risiko von ARGs in Trinkwassersystemen mit wenigen oder keinen Behandlungsschritten konnte nicht beobachtet werden, wobei sich zeigte, dass der wirksamste Behandlungsschritt zur Reduzierung von HNA Bakterien durch Infiltration von Oberflächenwasser in das Grundwasser erfolgt. In Zukunft müssten jedoch diese Ergebnisse in anderen Studien, die auf ein breiteres Spektrum von ARGs abzielen, weiter untersucht werden.

List of contents

List of abbreviations

ARG	-	antibiotic resistance genes
D.N.Q	-	detected but not quantifiable
dsDNA	-	double-stranded DNA
DWDS	-	drinking water distribution system
DWTP	-	drinking water treatment plant
Eawag	-	Swiss Federal Institute of Aquatic Science and Technology
EWL	-	Energie Wasser Luzern
FCM	-	flow cytometry
GAC	-	granular activated carbon
HNA	-	high nucleic acid
HPC	-	heterotrophic plate count
IWB	-	Industrielle Werke Basel
LNA	-	low nucleic acid
LOQ	-	limit of quantification
N.D	-	not detected
qPCR	-	quantitative polymerase chain reaction
TBW	-	Technische Betriebe Weinfelden
TCC	-	total cell count
TGB	-	Technische Gemeindebetriebe
UF	-	ultrafiltration
UMB	-	ultramicrobacteria

1 Introduction

Drinking water is one of the most important resources for human beings and should therefore be available to everyone in best quality. However, the increase of anthropogenic activities, including agricultural land use, livestock farming, landscape fragmentation, sewage and runoff discharge or natural causes has led to an continuing global aggravation of the water quality (Santos et al., 2019; Xu et al., 2020; Zhang et al., 2019). Accordingly to this, it is important that drinking water is purified by drinking water treatment plants (DWTP) in such a way, that the end user can drink this water without any concerns (World Health Organization, 2002). Although DWTPs involve several purification processes, including primary sedimentation, ultrafiltration (UF) membranes and granular activated carbon (GAC) biofiltration followed by disinfection with ozonation or chlorination and final ultraviolet (UV) light purification, some microbes may survive and flow into the drinking water distribution system (DWDS) (Besmer & Hammes, 2016; Goulas et al., 2020; Hou et al., 2018; Li et al., 2020; J. Liu et al., 2018; Zhang et al., 2019). In a recently published study, Hou et al. (2018) detected some genera such as *Pseudomonas*, *Citrobacter* and *Acinetobacter* as the dominant bacteria in treated water samples from a DWTP in South China containing also other potential human pathogens (*Enterococcus*, *Legionella*, *Mycobacterium*, *Salmonella*, *Staphylococcus*, *Streptococcus*). However, in European countries strict standards ensures the safety of drinking water and for Switzerland the drinking water has even the level of quality that can compete with that of mineral water (Blanc & Schädler, 2014). Nevertheless, the presence of potential human pathogens could increase the risks of water-related health problems (Suthar et al., 2008). Furthermore, Schwartz et al. (2003), amplified some antibiotic resistance genes (ARGs) (*vanA* and *ampC*) from the genomic DNA isolated from biofilms of a public drinking water distribution system in the city of Mainz (Germany). These ARGs indicate the presence of antibiotic resistant microorganisms. Although no cultivable bacteria linked to these ARGs could be found, the amplified genes may have been part of the genome of viable but non-cultivable aquatic bacteria (Schwartz et al., 2003). Bacteria getting in contact with a non-lethal dose of antibiotics are capable of different mechanisms to acquire ARGs or transfer them to other bacteria, making them resistant to antibiotics (Munita & Arias, 2016; Wright, 2011). As the use of antibiotics in medicine, livestock farming and agriculture more and more increases, antimicrobial resistance has become a serious threat to human health (Berendonk et al., 2015; Huang et al., 2016; Humphreys & Fleck, 2016; Leung et al., 2011). In case of ARGs in drinking water, it is important not only to monitor the DWTP processes, but also focus on the DWDS to estimate bacterial regrowth as a potential risk for human wellbeing and the spread of ARGs (LeChevallier et al., 1996; Niquette et al., 2001).

For these reasons, and in the event of a deterioration in water quality that could lead to a serious threat, further investigations and monitoring of DWTP and their distribution systems are essential (Goulas et al., 2020; Huang et al., 2016; Santos et al., 2019).

Bacteria represent the smallest form of independent life and bacteria with ultra-small size are ubiquitous in aquatic ecosystems, including oceans, rivers, lakes and groundwater (J. Liu et al., 2018; Proctor et al., 2018). Since 1993, different terminologies and names were used for bacterial groups with an ultra-small size, like «ultramicrobacteria» (UMB), «uncultivable bacteria» or «oligotrophs». However, with use of flow cytometry (FCM) in aquatic microbiology, two new terms have been assigned to planktonic bacteria (Wang et al., 2009). Starting with the terms «Group I cells» and «Group II cells» and changed to a couple of other names, Lebaron et al. (2001) named this groups in 2001 «low nucleic acid (LNA) content bacteria» and «high nucleic acid (HNA) content bacteria», which have since then been most widely used by researchers (Wang et al., 2009). According to Liu et al. (2018), UMB are defined as bacteria with small genomes (0.58 – 3.2 Mb) and a constant cell volume of less than 0.1 μm^3, whereas, as mentioned by Proctor et al. (2018), the upper limit is an order of magnitude smaller than a typical ***Escherichia coli*** cell (1 μm^3). Luef et al. (2015) detected ultra-small size bacteria with an average cell volume of 0.009 ± 0.002 μm^3 by electron microscopy, which might be the minimum viable cell volume required for life (National Research Council, 1999).

Since Koch's proposal in 1881 (Weiss, 2005), the heterotrophic plate count (HPC) method was used over a century for general microbiological monitoring of drinking water, but technology evolves and new methods are capable to replace HPC for microbiological monitoring (Van Nevel et al., 2017). One of these new methods is FCM and in a comparison to HPC data in several drinking water studies, FCM showed several advantages over HPC measurements for future researches (Gillespie et al., 2014; Hammes et al., 2008; Hoefel et al., 2003). On the other hand, HPC methods are relatively low cost and HPC data can be compared to more than a century of historical data worldwide. Nevertheless, Van Nevel et al. (2017) recently argued, that FCM cell counting is a suitable alternative to replace HPC for routine microbiological drinking water monitoring for several reasons. First of all, from about 15 water samples or more per day, FCM costs are equal to those of HPC and even more important, FCM detects all bacteria that are present, while HPC detects considerably less than 1% of the total bacteria and is often not detecting the dominant species (Van Nevel et al., 2017). Furthermore, Van Nevel et al. (2017) concluded that FCM provides the same relevant information as HPC for the same application areas in drinking water treatment processes, but is faster and more flexible.

In addition, the FCM data can be used to create a unique fingerprint of the bacterial community and offers simple automation options. Consequently, it is clear that FCM will increase in future studies and will be used for drinking water monitoring. Using FCM, a bimodal distribution of cells is often observed - based on a derived correlation between observed fluorescence intensity and cellular DNA/RNA content - where two dominant cell clusters are separated, representing the two groups of HNA and LNA content bacteria (Proctor et al., 2018). Wang et al. (2009) and Proctor et al. (2018) showed in their studies that HNA and LNA bacteria can be separated essentially by 0.45 μm membrane filtration to capture HNA bacteria, while the filtrate is filtered again with a 0.2 μm membrane filter to capture the LNA bacteria. Nonetheless, Wang et al. (2008) found that up to 10% of the microbial community were able to pass through 0.22 μm cartridge filtration units. Even though, the definition of LNA in this study are cells that could pass through 0.45 μm, but not through 0.2 μm filters, while any cells passing the 0.2 μm filters are not considered.

A currently unpublished research study from Eawag (the Swiss Federal Institute of Aquatic Science and Technology, Kastanienbaum, Switzerland) investigated antimicrobial resistances in HNA and LNA bacteria in water samples from a wastewater treatment plant in Switzerland and the receiving river. Their preliminary test showed that the proportion of the distribution of antibiotic resistance genes in the fraction of LNA bacteria is less than 3 % for the sulfonamide resistance gene ***sul1*** and as less than 1% for the erythromycin and tetracycline resistance genes (***ermB*** and ***tetW***) and for the class 1 integron-integrase gene (***intI1***). Considering to the fact that LNA content bacteria in a preliminary test of treated wastewater carried less than 1 - 3% of the ARGs investigated, the question arises, how important LNA content bacteria are with regard to antimicrobial resistance and its spread in DWDSs. There is a possibility that a minimum genome size for carrying an ARG could exists. The class 1 integrons are typically mobile genetic elements and frequently associated with aggregation of multiple antibiotic resistance genes and were therefore defined by Liao & Chen (2018) to be markers of the process of horizontal gene transfers. Sulfonamide, macrolide, trimethoprim, tetracycline, beta-lactam and quinolone plasmid-mediated resistance genes are frequently detected in different treatment processes and are reported to have significant correlations with the predominant class 1 integron-integrase gene ***intI1*** (Liao & Chen, 2018). Moreover, with a collective acquisition in the cassette array of over 130 different antibiotic resistance gene cassettes, the ***intI1*** gene has key advantages as a generic marker of anthropogenic influence to the environment (Gillings et al., 2015).

This study therefore focuses - within the context of an ongoing national founded project (NFP72) at Eawag, the «Swiss River Resistome» project, supported by the «Forschungsfond Wasser» (FOWA) of the «Schweizerischer Verein des Gas- und Wasserfaches» (SVGW) - on antibiotic resistances in HNA and LNA bacteria in water samples from several DWTPs and DWDSs in Switzerland.

One of the goals is to study the dynamics of HNA and LNA in different treatments of DWTPs and to compare the effectiveness of bacterial removal, especially in the case of antibiotic resistance genes in HNA and LNA content bacteria. The aim is to identify potential associations of HNA and LNA cells with antibiotic resistance indicator genes - the class 1 integron-integrase gene *intI1* and the ARG *sul1*. Furthermore, to study the distribution of ARGs in HNA and LNA content bacteria and the bacterial regrowth inside the DWDS due to different water sources and treatment steps and additionally, to investigate drinking water facilities with fewer or no treatment steps, to evaluate if they could represent a potential larger risk in case of ARGs or regrowth. FCM and other molecular methods such as quantitative PCR (qPCR) are used to see whether HNA and LNA content bacteria from drinking water sources and drinking water are associated with antibiotic resistances and to provide information to what extent FCM measurements can help to assess microbial risk factors. The establishment of such an association of LNA and HNA content bacteria with specific risk factors would increase their value as a diagnostic tool, especially in the drinking water sector.

2 Methods

Several DWTPs (three larger and three smaller sites, listed in Annex 1) all over the German speaking part of Switzerland were selected for sampling during summer season, with two different criteria. The main interest was on the drinking water source, which was primarily focused to be influenced by river water. The second criteria, was to compare larger DWTPs with several treatment steps against smaller DWTPs without any or fewer treatment. The smaller sites are not necessarily small in size but have fewer or no treatment steps while the larger sites treat their water with a combination of different steps.

After sampling, the water was stored at 4 °C and filtered on the next day. While filtering, the FCM was also done within 24 hours after sampling. The filters were stored at -20 °C for further testing. DNA extraction was done after all water samples from all sites have been filtered. Afterwards the quality of the DNA was measured with NanoDrop and Qubit to later test the antibiotic resistance with real-time qPCR.

2.1 Sampling

In Basel they use water from the river Rhein, whereas the DWTPs in Luzern uses the water from the lake Vierwaldstättersee in one plant and in the other plant groundwater mixed with spring water from catchment areas around the mount Pilatus. All three larger DWTPs have treatment steps like sand filtration, ozonation, activated charcoal filtration and UV-treatment or chlorination. The other smaller sites are Winterthur, Weinfelden and Bischofszell without or with single treatment steps. They take the groundwater, which is infiltrated from the river Töss in Winterthur and from the river Thur in Weinfelden and Bischofszell and distribute this water directly to the households (Figure 9, Figure 12 and Figure 15). The samples were taken from raw water, pumped groundwater within the well and from an end of pipe point, which is more or less one of the most distant points within the DWDS. The distribution times, inside the DWDSs to this end of pipe points, are listed in Annex 1. For the larger plants, some other samples of interest between the treatment steps were taken as well. For each sample point an amount of 35 litres were filled into autoclaved bottles and a canister, which was washed three times with alcohol, three times with tap water and three times with distilled water. On site, each of them was washed again three times with the sampling water before filled up. As the weather condition is an important factor for sampling, especially for the raw water from the river, the weather conditions for all sites can be viewed in Annex 1. Dry conditions were selected whenever possible.

IWB - Basel

The DWTP in Basel led by «Industrielle Werke Basel» (IWB) distributes drinking water to 210'000 people and is somehow unique, because the raw water from the river Rhein is pre-treated with a rapid filtration and then spread to some protected forest fields to let the water infiltrate into the groundwater before further treatment (Figure 1). This is followed by an activated charcoal filtration and finally UV-disinfection.

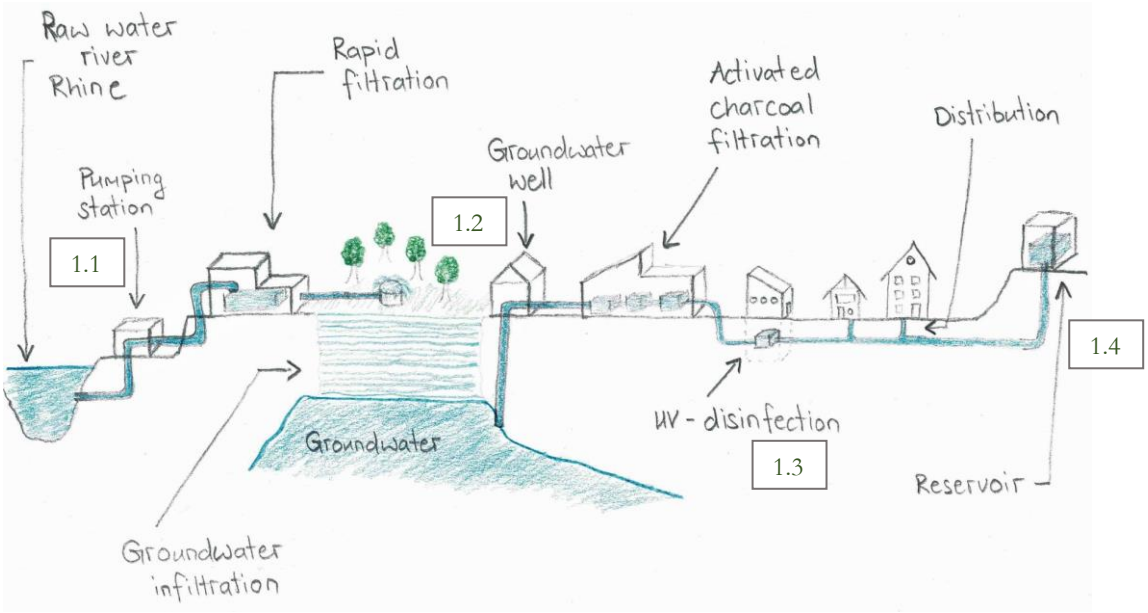


Figure 1: Overview of the DWTP in Basel with numbered sampling locations (Annex 1). (Source: Erb Severin)

Figure 2: Sampling site for raw water with a tap at IWB Basel. (Source: Erb Severin)

Raw water was taken inside the pumping station for the river Rhein (Figure 2), followed by a sample from the well were the groundwater is pumped. As a third point, the water after UV-disinfection was also measured and at last, some water from a well in Basel (Wasserturm-Brunnen) was chosen as end point sample of the DWDS (Figure 3). The drinking water needs approximately two to three days inside the DWDS to reach this well.

Figure 3: From left to right; Sampling at the ground water pumping station at IWB Basel (Source: Windisch Rainer, IWB); Sampling after UV-disinfection at IWB Basel; Sampling at the «Wasserturm-Brunnen» in Basel. (Source: Erb Severin)

EWL – Luzern

The drinking water in Luzern for about 110'000 people consists of water from three different sources. One of them is lake water from the lake Vierwaldstättersee, which is treated with ozonation (approximately 0.2 mg O_3/L), followed by activated charcoil and quartz sand filtration. In the end, a chlorination step is added befor the water is distributed (Figure 4). About 40% of the drinking water in Luzern is from lake water. The other 60%, treated in another DWTP also led by «Energie Wasser Luzern» (EWL) with similar treatment steps (Figure 5), are made up of about 50% spring water from the catchment areas Entlebuch and Eigental and 10% groundwater. Whereas groundwater was not examined because the groundwater source was not in operation during the sampling period. These two plants distribute their water to the DWDS simultaneous and therefore, the water will be mixed up somewhere in the middle between this two facilities. Both facilities were sampled to test if there are some differences between the water sources.

Raw water was taken directly from the pumping station. Another was taken after ozonation and one after final treatment, before the water enters the DWDS (Figure 6). In Figure 7, the other sample sites from the plant with spring and groundwater are visible. The groundwater pump would only be started if more water as usual is needed. The ozonation (around 0.2 mg O_3/L) in this DWTP is split up into three pipes. Because of this, the sample was taken as a mixture of approximately one third of each pipe. The end of pipe sample was chosen for both facilities on the same spot (Figure 8). The water needs about one day inside the DWDS to reach this end point.

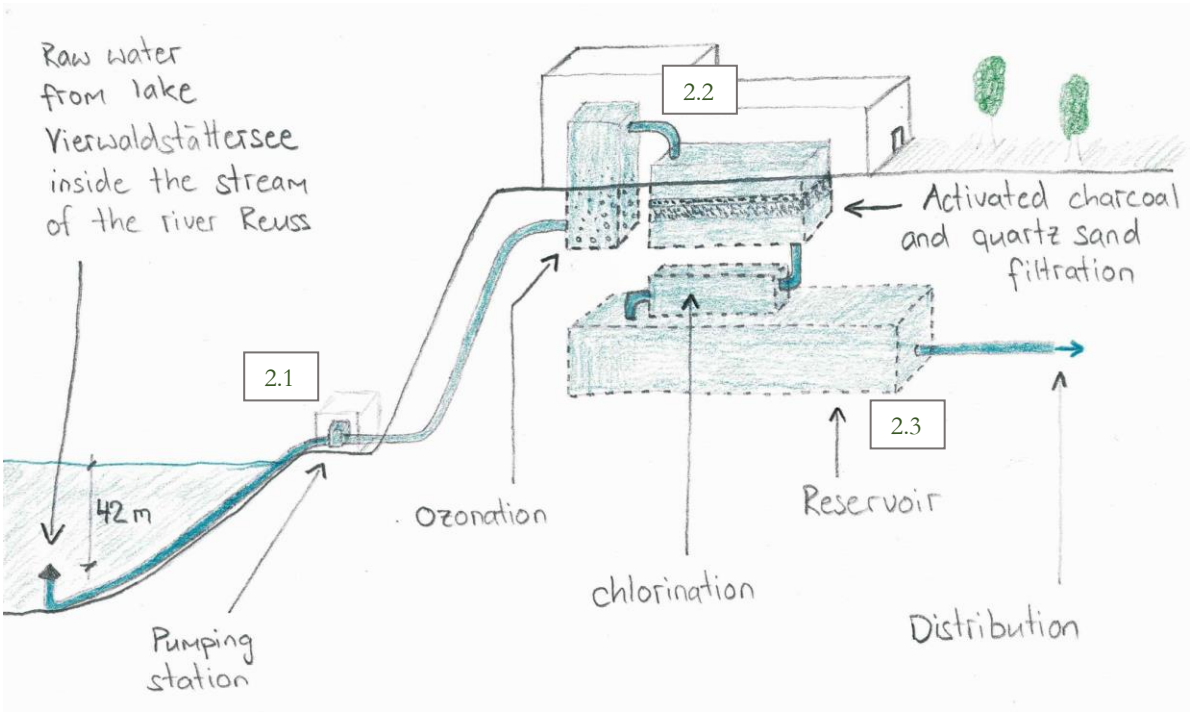


Figure 4: Overview of the DWTP in Luzern (lake water) with numbered sampling locations (Annex 1). (Source: Erb Severin)

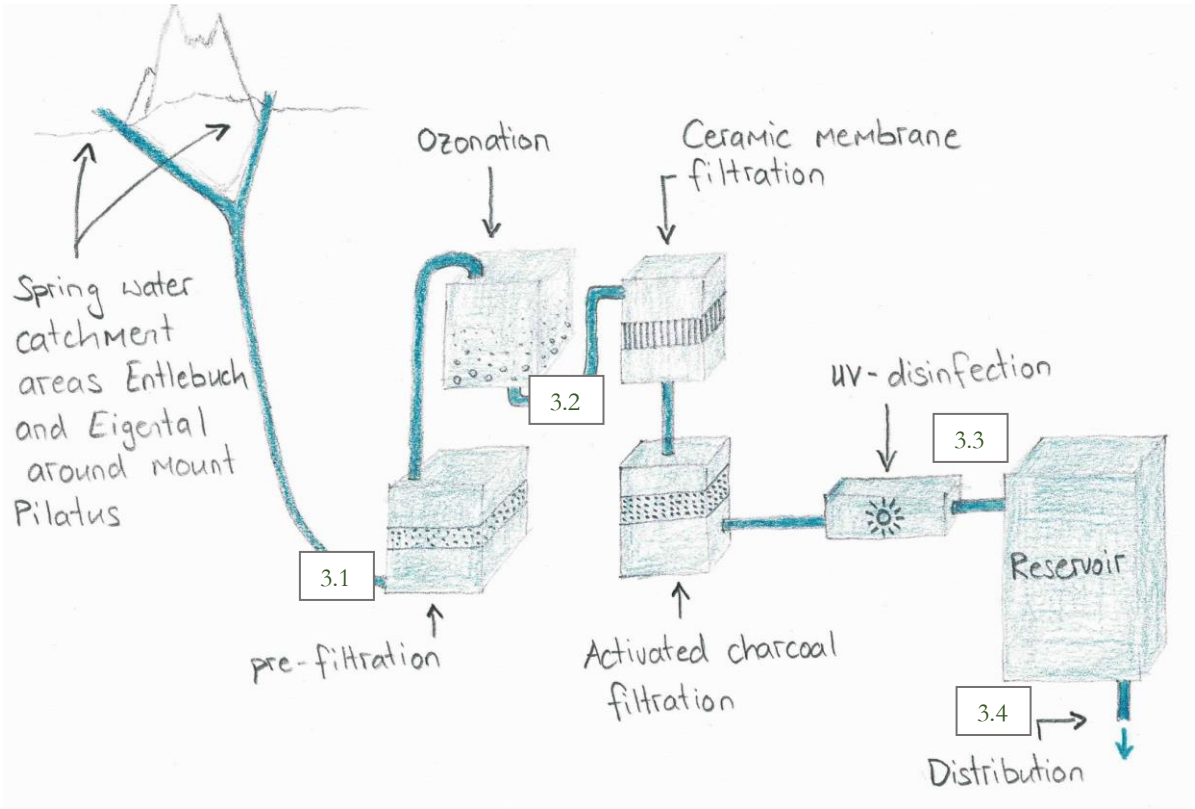


Figure 5: Overview of the DWTP in Luzern (spring water) with numbered sampling locations (Annex 1). (Source: Erb Severin)

Figure 6: From left to right; Raw water from lake Vierwaldstättersee; Sampling for water after ozonation; Final treated water before distribution. (Source: Erb Severin)

Figure 7: From left to right; Raw spring water from mount Pilatus; One of the sampling pipes for water after ozonation; Final treated water before distribution. (Source: Erb Severin)

Figure 8: Sampling site in Horw for the end of pipe water for both facilities in Luzern. (Source: Erb Severin)

Stadtwerk Winterthur

Stadtwerk Winterthur delivers drinking water for Winterthur and eleven neighbouring municipalities. The water is pumped out of the groundwater stream from the alpine river Töss and is clean enough to distribute without treatment. Water samples were taken from one pumping station near the river Töss, inside the groundwater protection zones, and another from a reservoir that delivers about three quarters of the water inside the DWDS. Four different pumping stations pump their water into this reservoir, where also a fifth pump can be started, which is located inside an agricultural landscape, used for feeding cows and growing crops. All five pumps were active during sampling and giving therefore a mixture of all possible sources for the reservoir. Special about this DWDS is the fact, that no energy is used to distribute the water, because the system can work with the natural slope that is given in Winterthur. Even more unusual is the water power turbine inside the reservoir, producing some amount of energy due to the slope between the pumping stations and the reservoir (Figure 9).

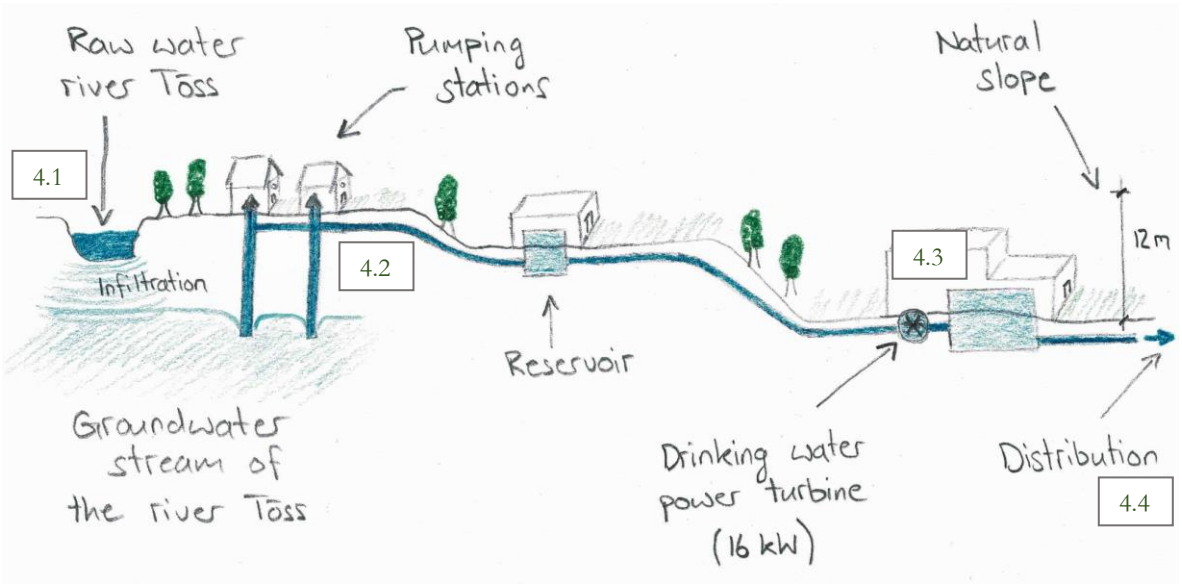


Figure 9: Overview of the DWTP in Winterthur with numbered sampling locations (Annex 1). (Source: Erb Severin)

To test the other quarter of the drinking water, one of the pumping stations near the river Töss was sampled. In Figure 10, the two sampling sites and the end of pipe site are visible. For the end of pipe sampling, one of the wells inside Winterthur was chosen. The water needs approximately about 1.5 days inside the DWDS to reach this well. Raw water was taken directly from the river Töss (Figure 11).

Figure 10: From left to right: Groundwater pumping station near the river Töss; Sampling site of the reservoir «Hornsäge/Hornwiden» in Winterthur; Sampling site for the end of pipe sampling at one of the wells inside Winterthur. (Source: Erb Severin)

Figure 11: Raw water sampling directly from the river Töss in Winterthur. (Source: Erb Severin)

TBW - Weinfelden

Drinking water in Weinfelden is provided from the groundwater stream of the river Thur. The river infiltrate into the groundwater of the pumping station and therefore directly influence the groundwater source. Nevertheless, the water is clean enough to distribute it to the households without any treatment step (Figure 12). «Technische Betriebe Weinfelden» (TBW) distribute their drinking water to the whole area of Weinfelden (15.48 Km^2).

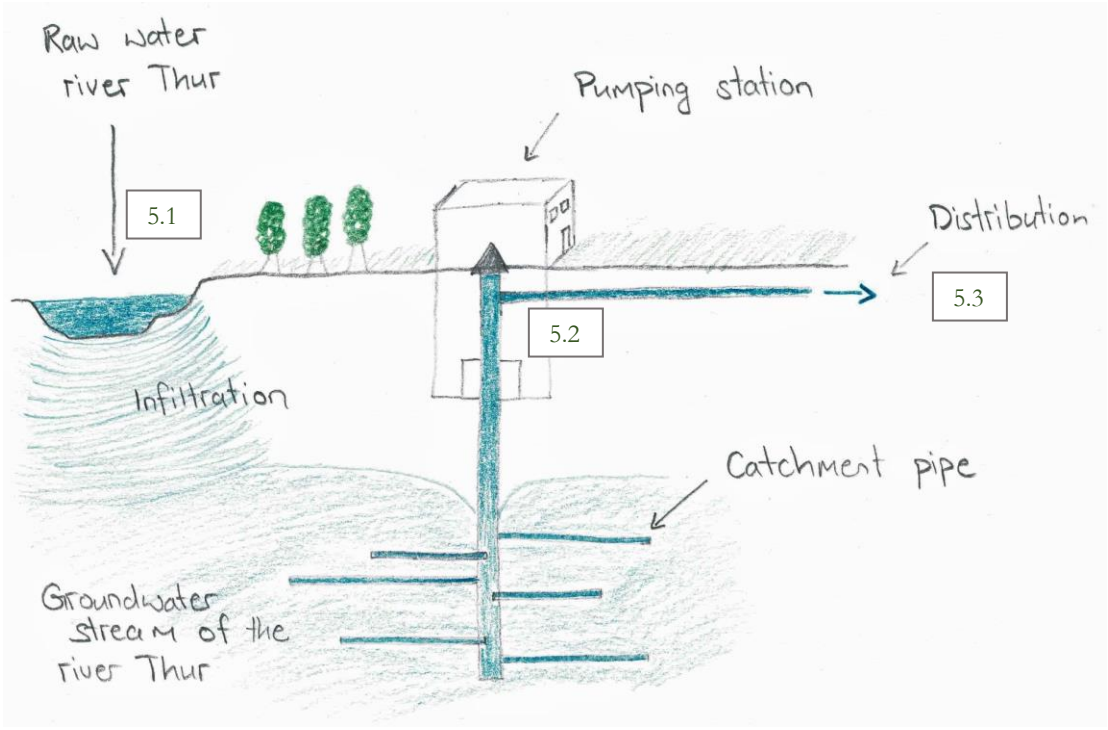


Figure 12: Overview of the DWTP in Weinfelden with numbered sampling locations (Annex 1). (Source: Erb Severin)

Figure 13 gives an overview of the groundwater pumping station, which was elected to take samples from. It shows the groundwater protection zones around the well and the sidearm river of the Thur, which flows near the well. The sampling point of the river is marked with a red circle. It is a rather low floating river, nearby the protection zone 2 (inner protection zone), one of the three groundwater protection zones in Switzerland, in which herbicides or pesticides are restricted. Raw water samples were taken out of the river with help of a bucket and funnel to fill up the canister (Figure 14). Figure 14 also shows the well that was chosen for an end of pipe sample. The water needs approximately half a day inside the DWDS to reach the well.

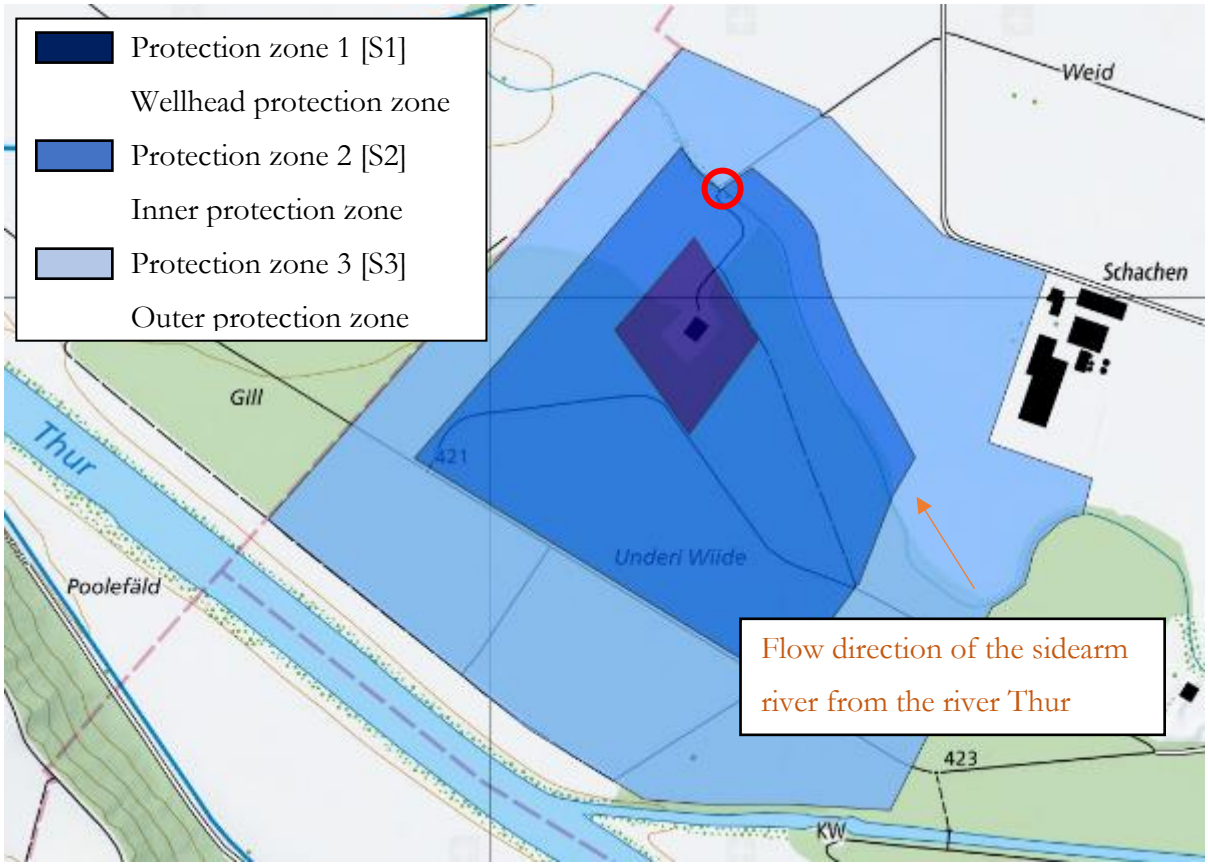


Figure 13: Overview of the groundwater pumping station in Weinfelden with all three protection zones. The sampling point for the raw water from the sidearm of the river Thur is marked with a red circle. (Source: FOEN, swisstopo, map.geo.admin.ch)

Figure 14: From left to right; Taking the raw water sample at Weinfelden; Drinking water well in Weinfelden for end of pipe sampling. (Source: Erb Severin)

TGB - Bischofszell

The drinking water for Bischofszell and three other neighboring municipalities is delivered by «Technische Gemeindebetriebe» (TGB) Bischofszell. Here, water is taken from the groundwater stream of the river Thur, the same river that is used in Weinfelden downstream of Bischofszell. At some points, the groundwater stream of the river Thur in Bischofszell flows just two meters below the surface. As single treatment step, a UV-disinfection is attached directly to the groundwater intake (Figure 15). The raw water sample was taken with help of a bucket directly from the river Thur and the one for the end of pipe sample out of a water hydrant (Figure 16), where the water had about two to three days retention time inside the DWDS.

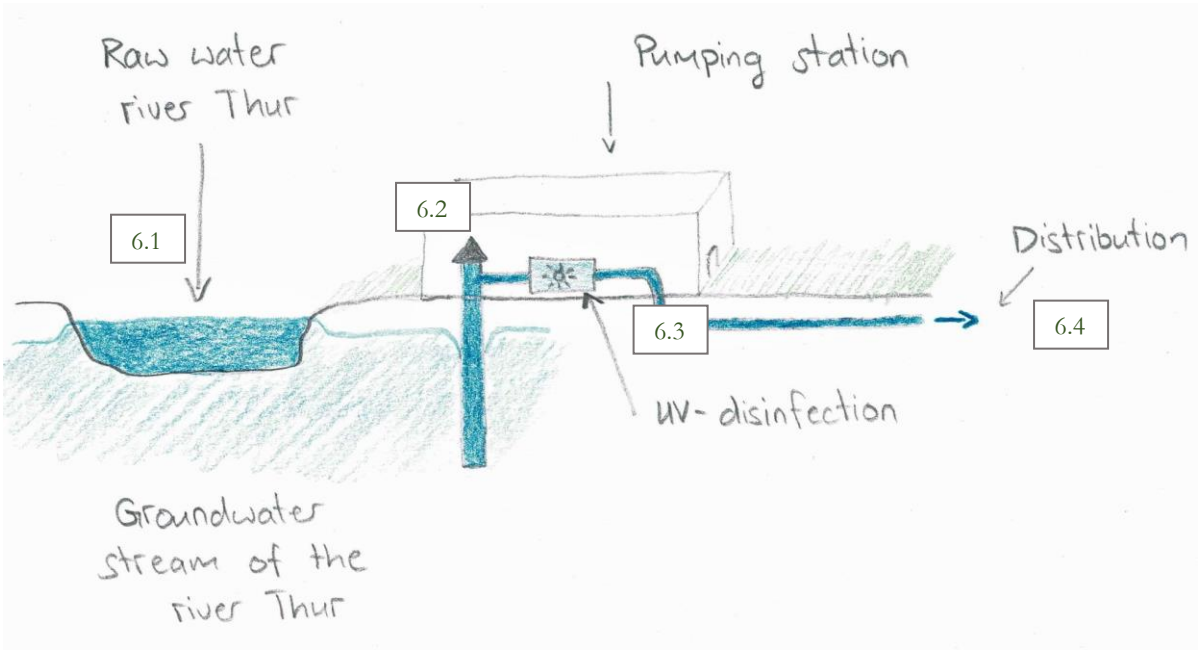


Figure 15: Overview of the DWTP in Bischofszell with numbered sampling locations (Annex 1). (Source: Erb Severin)

To test the groundwater and the drinking water after UV-disinfection, that enters the DWDS in Bischofszell, the groundwater pumping station «Grueben Niederbüren» was chosen. The two sampling sites which were selected for these samples are visible in Figure 17.

Figure 16: From left to right; Water sampling for raw water out of the river Thur; End of pipe sampling from a water hydrant in Bischofszell. (Source: Erb Severin)

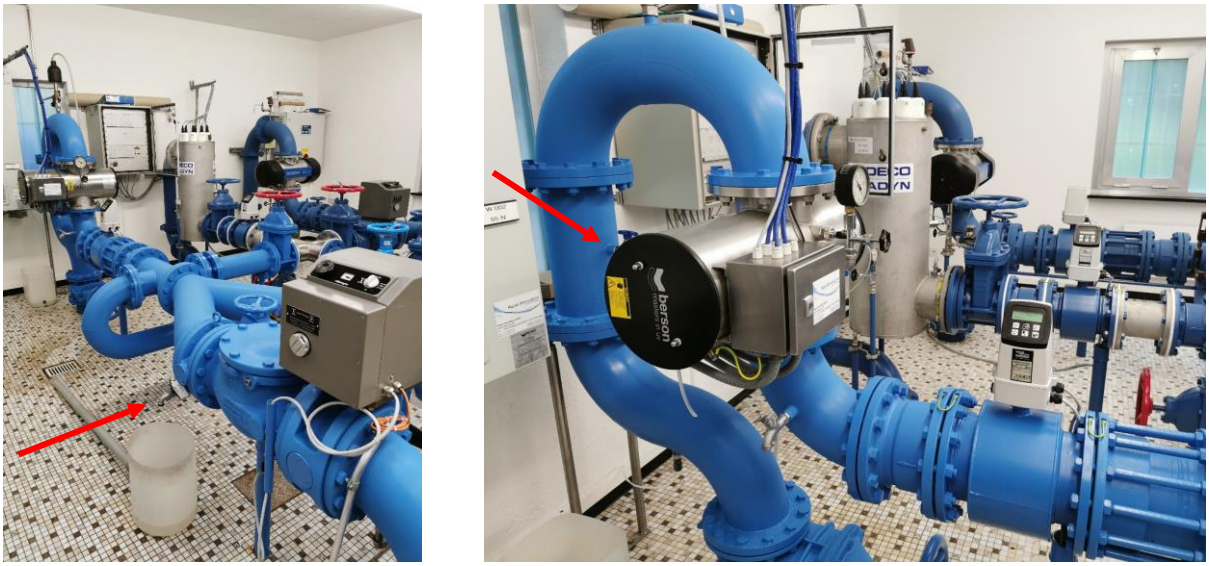


Figure 17: From left to right; Groundwater sampling site, marked with a red arrow, in Bischofszell; Sampling after UV-disinfection, marked with a red arrow. (Source Erb Severin)

2.2 Filtration of HNA and LNA content bacteria

Filtration of HNA and LNA content bacteria was done with nitrocellulose membrane filters type AC, SC (Sartorius Stedim Biotech GmbH, Germany). This technique was chosen, as the studies of Wang et al. (2009) and Proctor et al. (2018) showed, that filtration essentially separates HNA and LNA content bacteria. The samples were first filtrated directly on 0.45 µm membranes to capture HNA and 0.2 µm membranes for both HNA and LNA content bacteria as a comparison. Afterwards, the collected 0.45 µm filtered water was filtered again through 0.2 µm membranes to capture the LNA content bacteria. To catch enough DNA for further testing, a pre-test was done to estimate the amount of water, that was feasible to filtrate during this project due to clogging of the filters. A maximum amount of 10 litres for 0.45 µm and 0.45/0.2 µm and of 5 litres for direct filtration on 0.2 µm membranes resulted from the pre-test to be feasible for treated water, while clogging in raw water took place much faster. All filtered amounts are listed in Annex 1. The sampled water was filtered after a storage time of approximately 24 hours at 4 °C. The filtration setup where sampled water was filtered with autoclaved filter units through the membranes, while a pump produced a vacuum under the filter membrane to reduce the filtration time, is shown in Figure 18. The membranes were collected with sterile forceps after filtration and stored in plastic bags at -20 °C before DNA extraction (Figure 19).

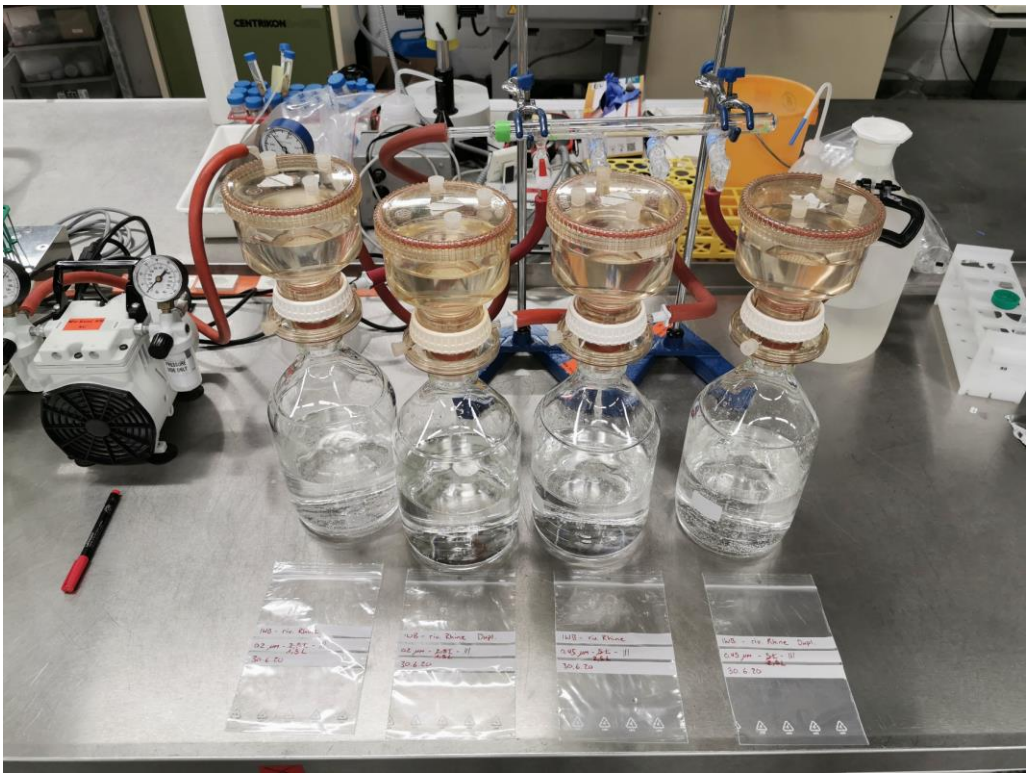


Figure 18: Filtration setup with autoclaved filter units and vacuum pump. (Source: Erb Severin)

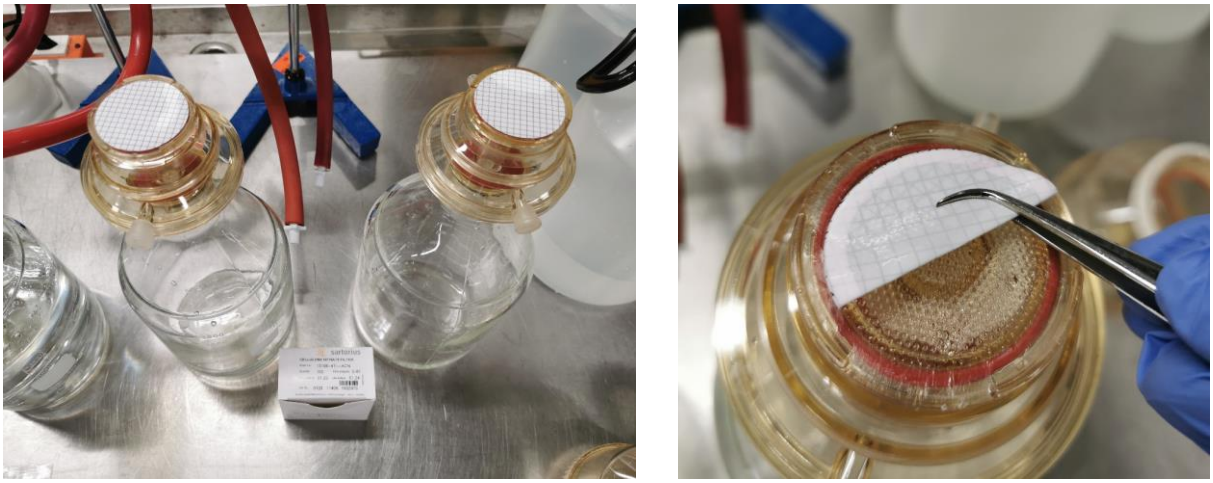


Figure 19: From left to right; Prepared filter units for filtration; Collecting a filter membrane after filtration. (Source: Erb Severin)

2.3 FCM

FCM has emerged as one of the most straightforward applications for detection of changes in drinking water quality or monitoring of treatment processes in determination of total cell counts (TCC) (Prest et al., 2013). The TCC method was applied with the officially accepted guideline for drinking water analysis in Switzerland (Kötzsch et al., 2012). A fluorescent stain (SYBR® Green I) which binds preferentially to nucleic acids after passing the membrane of bacteria – but also the membrane of other organisms - has been added to the sample (Prest et al., 2013). The measurements were performed using a BD Accuri C6® flow cytometer (BD Accuri cytometers, Belgium) equipped with a laser emitting a wavelength of 488 nm. The machine separates the cells during measurement by hydrodynamic focusing through a glass capillary, which is irradiated by a laser, installed in the middle of this capillary and irradiate horizontally through it. Each cell that passes the laser causes light scattering and fluorescent light emission, depending on the fluorescent dye used (Kötzsch et al., 2012). According to Kötzsch et al. (2012), two filters inside the FCM are installed to detect specific wavelengths. The side scattered laser light, when a cell is passing by (measuring background signal), is measured by a detector before the light passes through the first filter. According to Kötzsch et al. (2012), this filter captures the green fluorescence of SYBR® Green I at 520 nm (for TCC), while another detector counts the red fluorescence at 630 nm (for the «live/death» measurement with propidium iodide) of each cell, although the «live/death» measurement was not used in this study.

Staining protocol

The samples were stained according to the standardized protocol in the Swiss guideline for drinking water analysis as described by Kötzsch et al. (2012). They were pre-heated to 35 °C (5min) and stained with 10 µL ml-1 SYBR® Green I (1:100 dilution in dimethyl sulfoxide; Molecular Probes) for TCC measurement. The samples were then incubated in the dark for 10 - 15 min at 35°C before measuring (Kötzsch et al., 2012). Raw water samples have been diluted up to 1:40, depending on samples, in filtered bottled mineral water (0.22 µm; Millex-GP, Millipore filtered EVIAN mineral water, France) before staining. Furthermore, it is important to dilute the samples before staining, otherwise the dye could partially diffusing out of the cell, which would result in a lower signal (Kötzsch et al., 2012).

Gating strategy

For comparing the sample sites after measurement, it is necessary that the same gate is used for all of them. Otherwise, a conclusion between the sampling sites of the different DWTPs would not be scientifically verifiable. Therefore, the standard gate from Eawag (Kastanienbaum) was used for all FCM runs (Figure 20). This gate was set according to the guideline for drinking water analysis in Switzerland (Kötzsch et al., 2012).

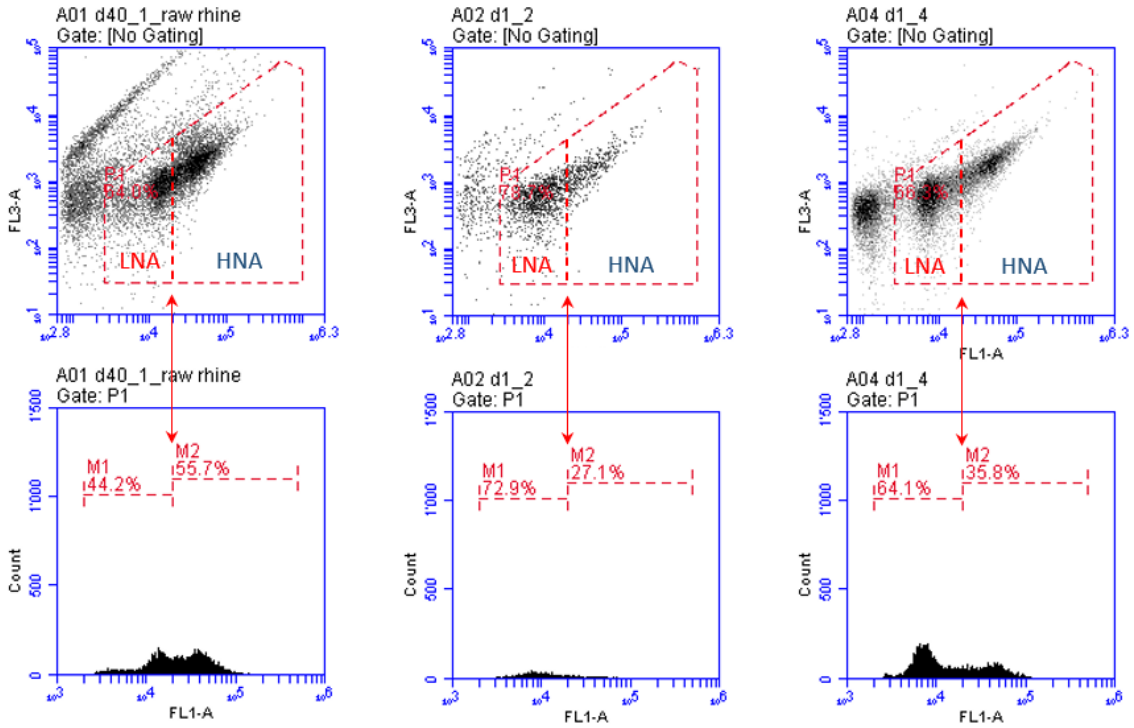


Figure 20: FCM results (SYBR Green I) for drinking water samples at the DWTP in Basel using the same gating strategy. From left to right; Raw water Rhein; Groundwater in Basel; End of pipe in Basel.

2.4 DNA extraction

Extraction of DNA has been conducted with a standard protocol of the DNeasy® PowerWater® Kit (QIAGEN, Netherlands) (viewable in Annex 2). The filters were unwrapped with sterile forceps, rolled into cylinders and inserted into 50 mL collection tubes. Together with beads and solution PW1, the tubes were beaten inside a beat machine for 40s with 6 m/s. This mechanical action of bead beating breaks the surface of the filter membrane apart that contains trapped cells and aids in cell lysis. After centrifugation and collecting the supernatant to another tube, the solution IRS was added, which contains a reagent to precipitate non-DNA organic material, including cell debris and proteins, for a better downstream application and higher purity of the extracted DNA. As next step, the tubes were centrifuged and the supernatant collected again into a new tube. Solution PW3 was then added, which is a high concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this adjusts the DNA solution salt concentrations to allow binding of the DNA, while preventing the binding of non-DNA material to the MB Spin column. The supernatant with solution PW3 was then added to a MB Spin column, containing a silica membrane that selectively binds DNA, whereas the flow-through with non-DNA components was discarded after centrifugation. With solution PW4 and ethanol, the DNA on the membrane was washed to removes residual salt and other contaminants while allowing the DNA to stay bound to the silica membrane. To collect the DNA from the washed membrane, solution EB was added to the MB Spin column and the tubes were centrifuged one last time. As solution EB passes through the silica membrane, the DNA that was bound in the presence of high salts was selectively released due to the EB solution, which does not contain any salts. The extracted DNA inside the EB solution was then ready for further analyses.
Afterwards, the extracted DNA was assembled from both duplications (Annex 1) to obtain more DNA for analysis with real-time qPCR.

2.5 NanoDrop and Qubit

To measure the amount of double-stranded DNA (dsDNA) inside the extracted DNA solution, NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) was used before other downstream applications were done. First of all, a blank has to be measured to set the calculation of absorbance. The machine measures the light that passes through the liquid sample of about 1.5 µL to the detector and shows the amount of light absorbed by the molecules at each measured wavelength. This sample intensities along with the blank intensities are used to

calculate the total absorbance, to correlate the sample absorbance with concentration in use of the Beer-Lambert law:

$$Absorbance = -\log\left[\frac{intensity_{sample}}{intensity_{blank}}\right]$$

$$Beer-Lambert\ law:\ A = \varepsilon * b * c$$

where:

A = absorbance in absorbance units (A)

ε = wavelength-dependent molar absorptivity coefficient in litre/mol*cm

b = pathlength in cm

c = analyte concentration in moles/litre or molarity (M)

The lower detection limit for dsDNA is 0.20 ng/µL, while the A260/A280 purity ratio is a ratio of corrected absorbance at 260 nm wavelength to corrected absorbance at 280 nm. A purity ratio of A260/A280 of about 1.8 is generally accepted as «pure», while an A260/A230 purity ratio between 1.8 and 2.2 is accepted as «pure» for DNA, whereas environmental DNA will normally not reach these purification levels.

Qubit measurements were done with a Qubit 2.0 fluorometer (Thermo Fisher Scientific, USA). The fluorometer detects fluorescent dyes that bind specifically to the target of interest with a detection limit for dsDNA of 0.001 ng/µL. Therefore, the measurement is more precise than NanoDrop for low amounts of DNA.

2.6 Quantitative PCR

Real-time quantitative polymerase chain reaction (real-time qPCR) has been used to investigate one ARG (*sul1*) and one integron-integrase gene (*intI1*), while to search for regrowth inside the drinking water system, a real-time qPCR for 16S rRNA was performed to investigate general bacteria. A LightCycler® 480 Instrument II (Roche, Switzerland) was used for all qPCRs. The primer sets are shown in Table 1 and the program settings in Table 2. All samples were diluted 1:10 with AE Buffer (QIAGEN, Netherlands) and raw water samples were diluted 1:100 as well for 16S rRNA. The standard dilution started with 50 million copies/2 µL and was serial diluted with AE Buffer until 50 copies/2 µL, which represents the lowest limit of quantification (LOQ). All samples were run in triplicate for more significant results.

Table 1: Used primer sets for real-time qPCR.

Primer	Target genes	Assay type	Sequences (5' to 3')	Annealing temp (°C)	Ref.
BAC349-F	16S rRNA	TaqMan	AGGCAGCAGTDRGGAAT	53	1*
BAC806-R			GGACTACYVGGGTATCTAAT		
BAC16F-Probe			*FAM*-TGCCAGCAGCCGCGGTAATACRDAG-*TAMRA*		
intI1-F	*intI1*	TaqMan	GCCTTGATGTTACCCGAGAG	60	2*
intI1-R			GATCGGTCGAATGCGTGT		
intI1-Probe			*FAM*-ATTCCTGGCCGTGGTTCTGGGTTTT-*BHQ1*		
qSUL653f	*sul1*	TaqMan	CCGTTGGCCTTCCTGTAAAG	60	3*
qSul719			TTGCCGATCGCGTGAAGT		
tpSUL1-Probe			*FAM*-CAGCGAGCCTTGCGGCGG-*TAMRA*		

1* (Takai & Horikoshi, 2000)

2* (Barraud et al., 2010)

3* (Heuer & Smalla, 2007)

Table 2: Program settings for LightCycler® 480 Instrument II (Roche, Switzerland).

Target genes	Program	T (°C)	Ramp time	Time [mm:ss]	Nr. of Cycles
16S rRNA	Init. Denature	95	4.4 °C/sec	10:00	
	Denature	95	4.4 °C/sec	00:40	45
	Anneal	53	2.2 °C/sec	00:40	
	Extend	72	2.4 °C/sec	01:00	
		40	2.5 °C/sec		
intI1	Init. Denature	95	4.4 °C/sec	10:00	
	Denature	95	4.4 °C/sec	00:30	45
	Anneal	60	2.4 °C/sec	01:00	
		40	2.5 °C/sec		
sul1	Init. Denature	95	4.4 °C/sec	10:00	
	Denature	95	4.4 °C/sec	00:15	45
	Anneal	60	2.4 °C/sec	01:00	
		40	2.5 °C/sec		

Results from real-time qPCR were compared in Excel to calculate the average Ct-value (copies/µL) and the standard deviation of the Ct-value for each measured triplicate. The lowest detection limit for each run (or the highest quantifiable Ct-value), which is classified as the lowest limit of quantification, was represented by the average Ct-value of the serial diluted standard curve with the lowest amount of copies/µL (50 copies/2 µL) (Table 3). Since a lot of the 1:10 diluted samples were below the LOQ, another qPCR run with undiluted samples was done for those samples who had a higher average standard deviation of the Ct-value than 0.5 (which would mean, that the result is not scientifically trustful) or had a higher average Ct-value than the lowest detection limit of the standard curve (the lower the limit the higher the Ct-value). Whereas some of the ***sul1*** samples below the LOQ and the same criteria, but with average Ct-values above the LOQ + 3.3, were not re-measured to save raw DNA for further analyses. Negative controls with and without H_2O were measured below the LOQ in all runs. However, even with undiluted samples, some of them could not be quantified or detected and were therefore categorized in two groups:

- **Detected but not quantifiable (D.N.Q)**
  The standard deviation of the Ct-value is higher than 0.5 and/or the average Ct-value of the sample is higher than the average Ct-value of the lowest detection limit of the standard curve (Table 3).
- **Not detected (N.D)**
  Two or more of the triplicates from the sample are not detected (have no Ct-value) or the average Ct-value is higher than the Ct-value of the negative control.

The average copies/mL was then calculated in Excel. The Ct-value was divided by 2 (because of the 50 copies/2 µL as lowest detection limit) to get to 1 µL and then multiplied by the elution volume (amount of solution EB used in the DNA extraction (Annex 2)). After that, it was divided again with the amount of water filtered through the membranes in mL (Annex 1) and multiplied by the dilution factor to get to the value «copies/mL» in the end. Therefore, the lowest detection limit depends also on the amounts of water that were filtered through the membranes, the more water filtered the lower is the detection limit, whereas 25 copies/µL (0.025 copies/mL) as the lowest detection limit of the standard curve was already low. The average of the total copies/mL for each site (normally the average out of three because of triplicates) and the standard error (calculated as standard deviation of the values divided by the square root of the amount of values used) was combined in a csv file to visualize the results in «R» using bar chart plots.

3 Results

3.1 NanoDrop and Qubit

Researchers at Eawag (Kastanienbaum) consider values below 15 ng/µL from NanoDrop not as trustful results. As just a few water samples had values higher than 15 ng/µL, all samples were cross-checked with Qubit fluorometer, which can measure much lower values and will give more accurate results. Nevertheless, NanoDrop provided the A260/A280 and A260/A230 ratios, which are necessary to see if the DNA extraction worked properly. The NanoDrop and Qubit results are shown in Annex 3.

3.2 FCM and quantitative PCR

The FCM and real-time qPCR results for all samples at each DWTP are given in the following chapters as bar chart plots made in «R». The measured efficiencies, slopes, Y-intercepts and the average Ct-value of the lowest detection limit of the standard curve, together with the standard deviation of the lowest detection limit for all qPCR runs, are shown in Table 3. The standard curve was measured in quintuplicates to calculate the average lowest Ct-value.

Table 3: Measured accuracy values for all real-time qPCR runs with the average lowest Ct-values of the standard curve and their standard deviation Ct-values.

Dilution	Target genes	Efficiency	Slope	Y-intercept	Avg. lowest Ct-value	St. dev. lowest Ct-value
1:10 and 1:100	16S rRNA	1.946	-3.459	41.17	50(32.22)	0.236
1:10	*intI1*	1.934	-3.492	41.16	50(34.39)	0.827
Undiluted	*intI1*	1.912	-3.554	41.48	50(34.42)	0.584
1:10	*sul1*	1.974	-3.387	39.18	50(33.55)	0.382
Undiluted	*sul1*	1.995	-3.335	38.81	50(33.29)	0.395

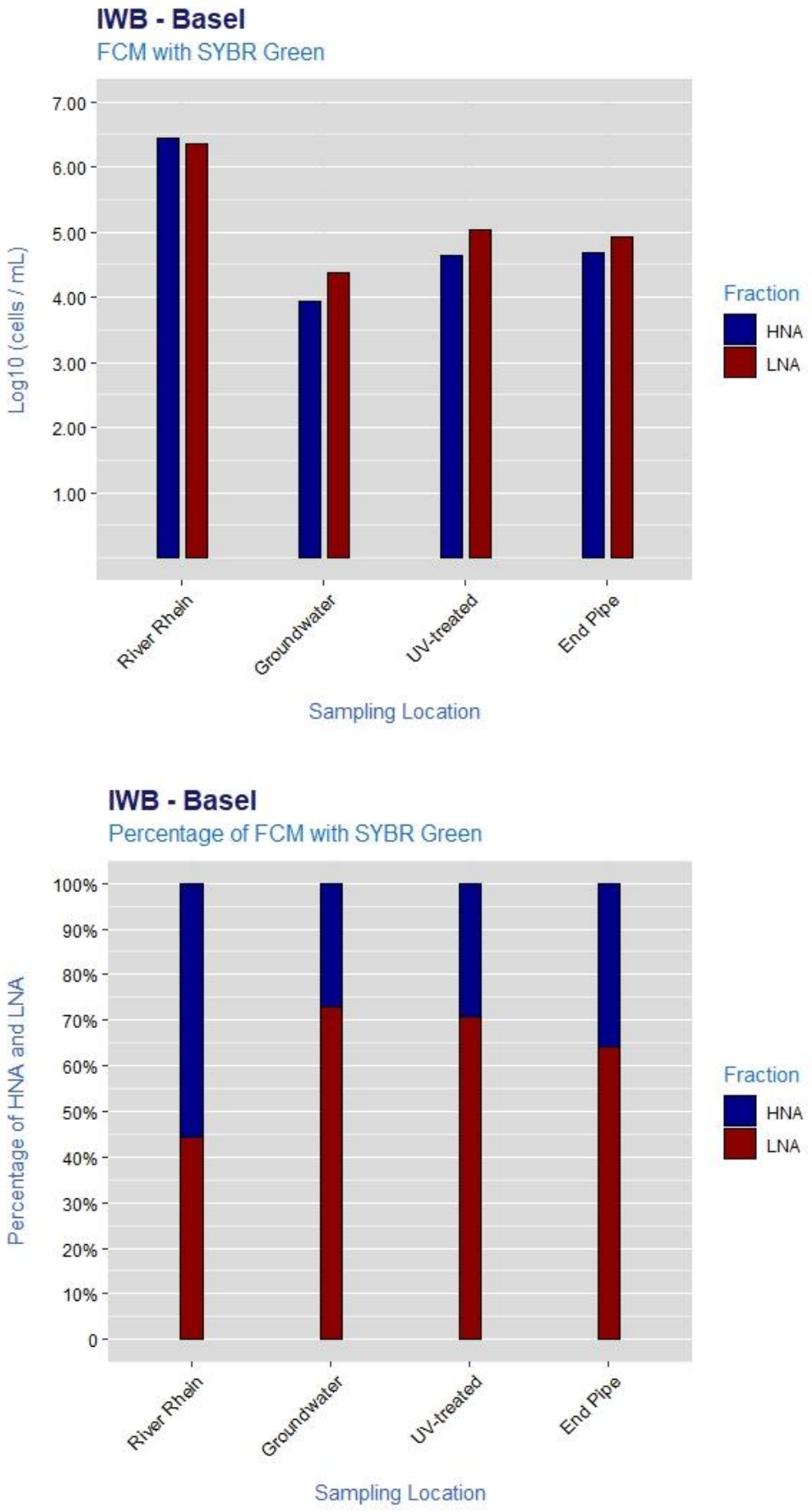
The «R» graphs are plotted in a log10-scale to give a better visualization of the data, except for sites with low copies (below 100 copies/mL). Green bars (filtered on 0.2 µm) representing HNA and LNA together, whereas blue bars (filtered on 0.45 µm) showing HNA and red bars (filtered on 0.45 µm leftovers filtered again on 0.2 µm) visualizing LNA. The orange bars indicate the measured standard error for each sampling location. Bars with no values are either marked as detected but not quantifiable (D.N.Q) or as not detected (N.D).

IWB – Basel

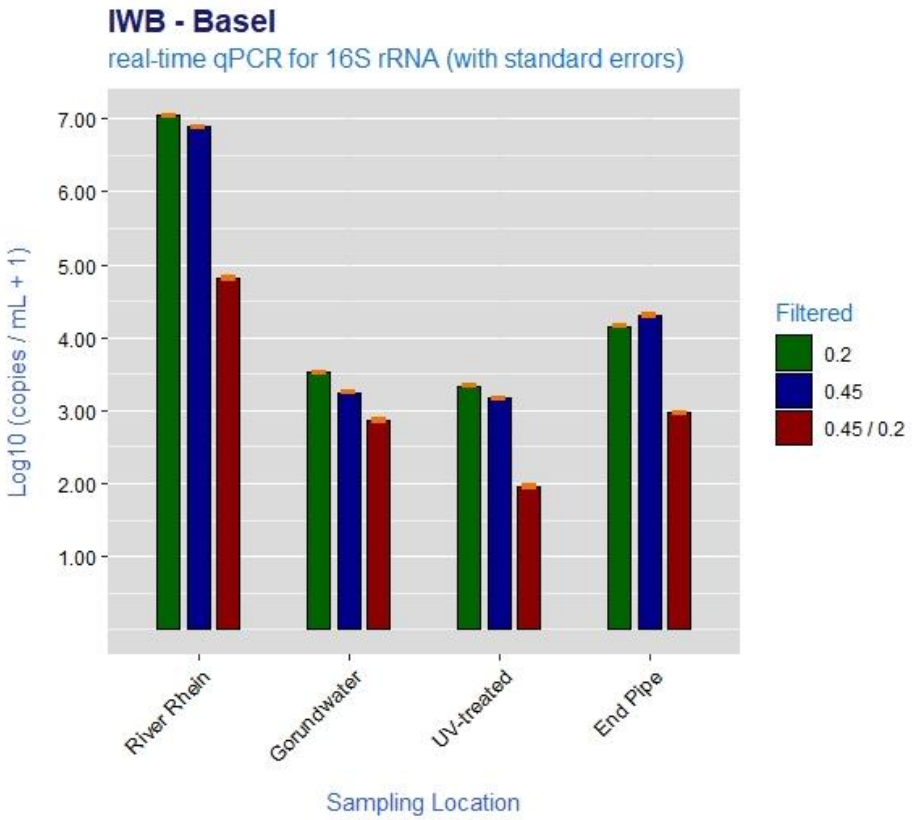
The FCM results from «Industrielle Werke Basel» (IWB) in R-plot 1 shows that the proportion of HNA and LNA cells in the river Rhein was almost half/half. The groundwater infiltration changed this proportion to more LNA than HNA and had more effects on the larger fractions, dropping the proportion of HNA cells by 30%. On the other hand, the total amount of bacteria decreased for both fractions after groundwater infiltration by around two log10-values. The sampling location for UV-treated water shows that there is a slight increase for both fractions, by a factor of close to six inside the DWTP after activated charcoal filtration and UV-disinfection, while the proportion of bacteria with HNA and LNA content stayed stable. The sample from the «Wasserturm-Brunnen» («End Pipe» sample) shows that the proportion of HNA content bacteria increased inside the DWDS by a few percent.

Real-time qPCR for 16S rRNA (R-plot 3) showed that HNA content bacteria between raw water from the river Rhein and groundwater infiltration decreased by three log10-values, while LNA decreased by two log10-values, which indicates, as shown above (R-plot 1), that the infiltration has a higher effect on bacteria with HNA content, whereas the treatment steps inside the DWTP had more effect on LNA content bacteria. Between the sampling points after UV disinfection (start of DWDS) and the end of the pipe, a regrowth by one log10-value is visible for both fractions.

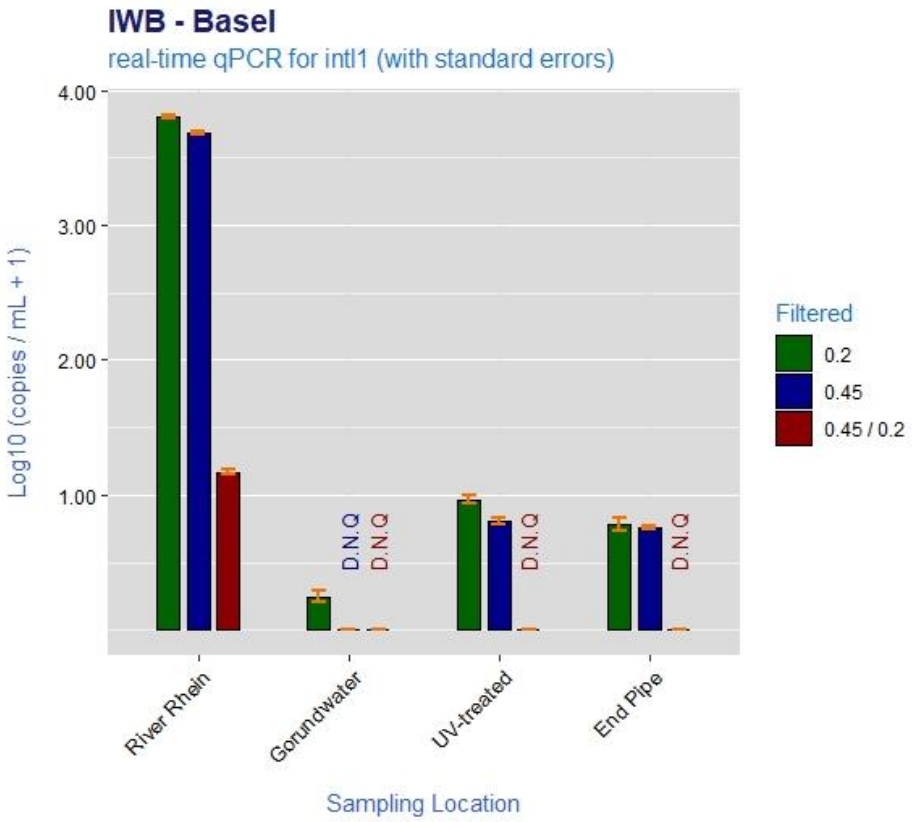
Targeting *intI1* and *sul1* revealed a larger presence in HNA content bacteria in the river Rhein samples (R-plot 2 and R-plot 4). The class 1 integron-integrase gene *intI1* was not quantifiable for bacteria with LNA content at all other locations, but was detected with an amount of approximately 4 copies/mL within HNA content bacteria. Additionally, no increase of *intI1* or *sul1* inside the DWDS could be measured. The ARG *sul1* was not detected in bacteria with LNA content in all samples except for raw water from river Rhein, but detected with a very low amount of about 1 copy/mL within HNA content bacteria of the «End Pipe» sample, whereas the detection inside the DWTP was not quantifiable.



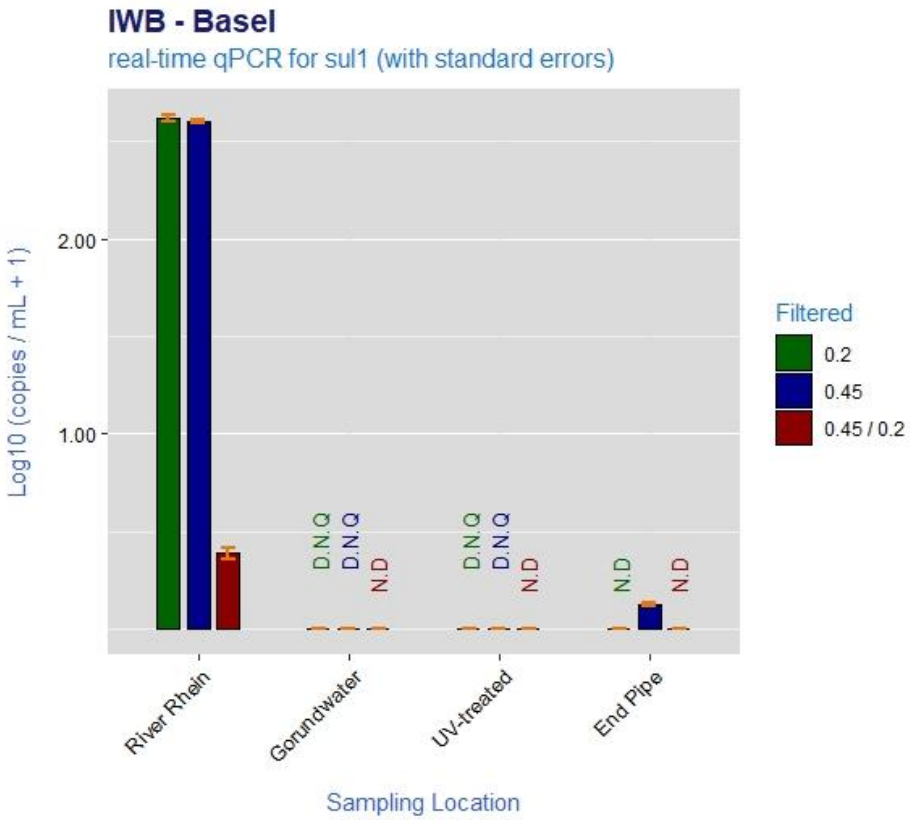
R-plot 1: FCM runs (SYBR Green I) for all sampling locations at the DWTP in Basel.



R-plot 3: Real-time qPCR for 16S RNA for all sampling locations at the DWTP in Basel.



R-plot 2: Real-time qPCR for *intl1* for all sampling locations at the DWTP in Basel.



R-plot 4: Real-time qPCR for *sul1* for all sampling locations at the DWTP in Basel.

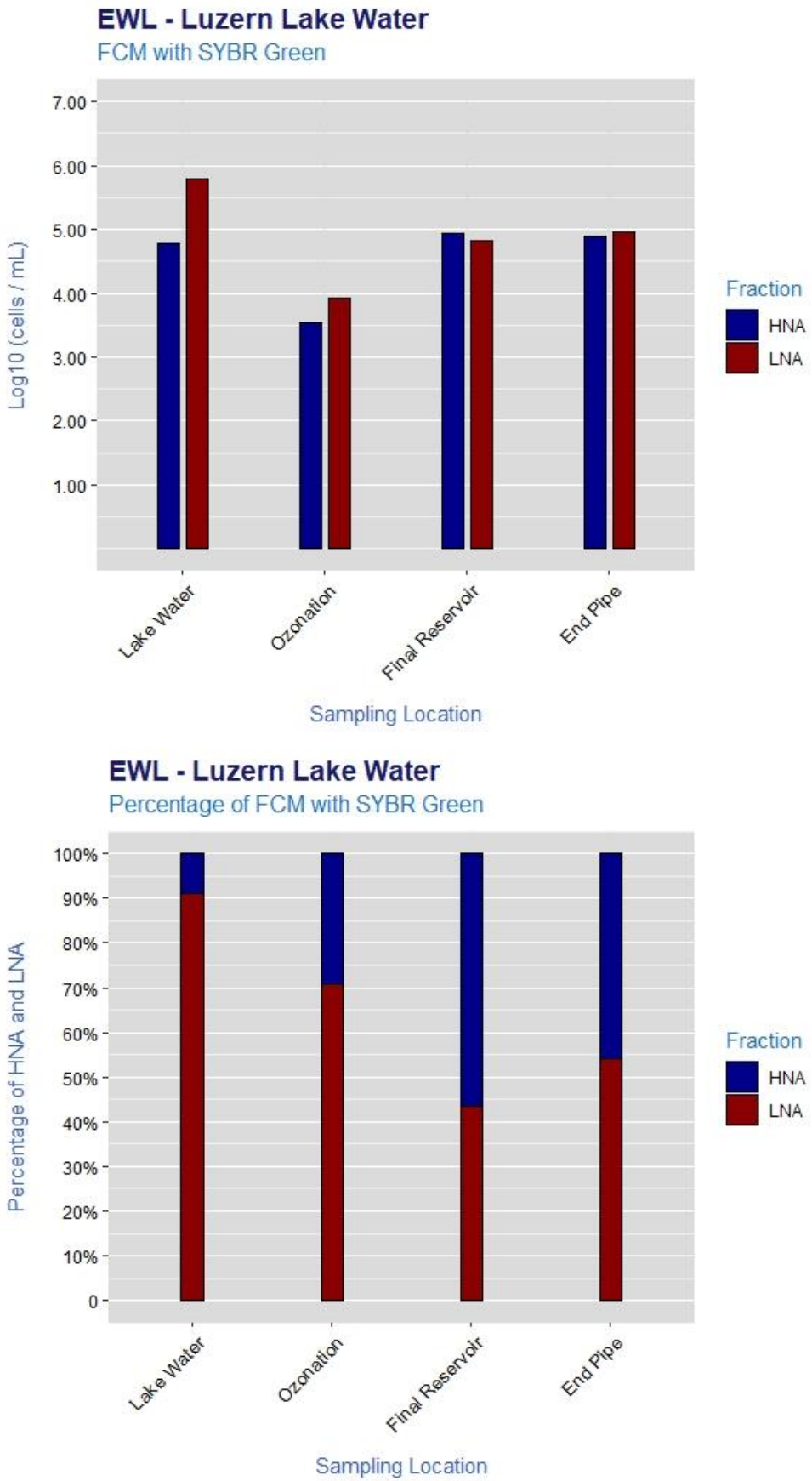
EWL - Luzern

The «End Pipe» location is the same for both DWTPs, led by «Energie Wasser Luzern» (EWL) (2.1). It can be said, that the DWTP using spring water influences the «End Pipe» location more than the other plant, due to the location of the plants in the DWDS of Luzern. The water from both plants is mixed together in the middle of the distribution, but since the plant using spring water is much closer to the «End Pipe» location, more water from this plant reaches the «End Pipe» sampling site.

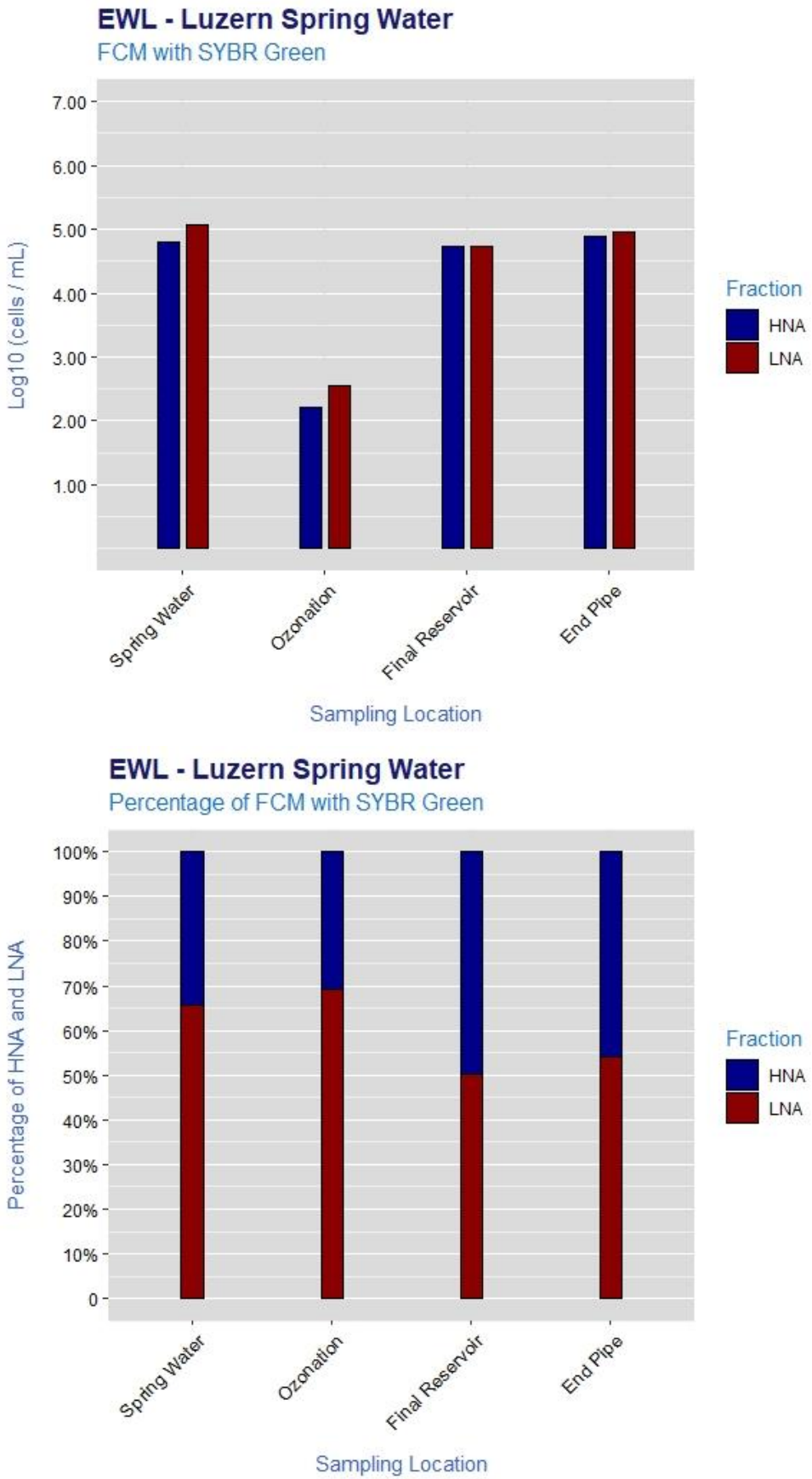
R-plot 5 and R-plot 6 shows that the percentage of LNA cells, compared to other raw water samples from rivers (Annex 1), is high in Vierwaldstättersee lake water (90%) and relatively high in spring water (65%). It is also visible, that the percentage of HNA increases within the DWTP using lake water (increase of 40%) more than within the other DWTP using spring water (increase of 15%). Compared to other «End Pipe» locations consisting of treated river water, the «End Pipe» sample in Luzern generally shows a larger proportion of HNA than all other tested sites.

Real-time qPCR for 16S rRNA (R-plot 7) showed a decrease of both fractions between raw water and ozonation by a factor of two log10-values, but also that there is a significant increase within the DWTPs (for spring water more than for lake water). The «Final Reservoir» samples have roughly the same amount of both fractions and regrowth inside the DWDS was low for bacteria with HNA content, while LNA content bacteria increased by a factor of about ten within both plants.

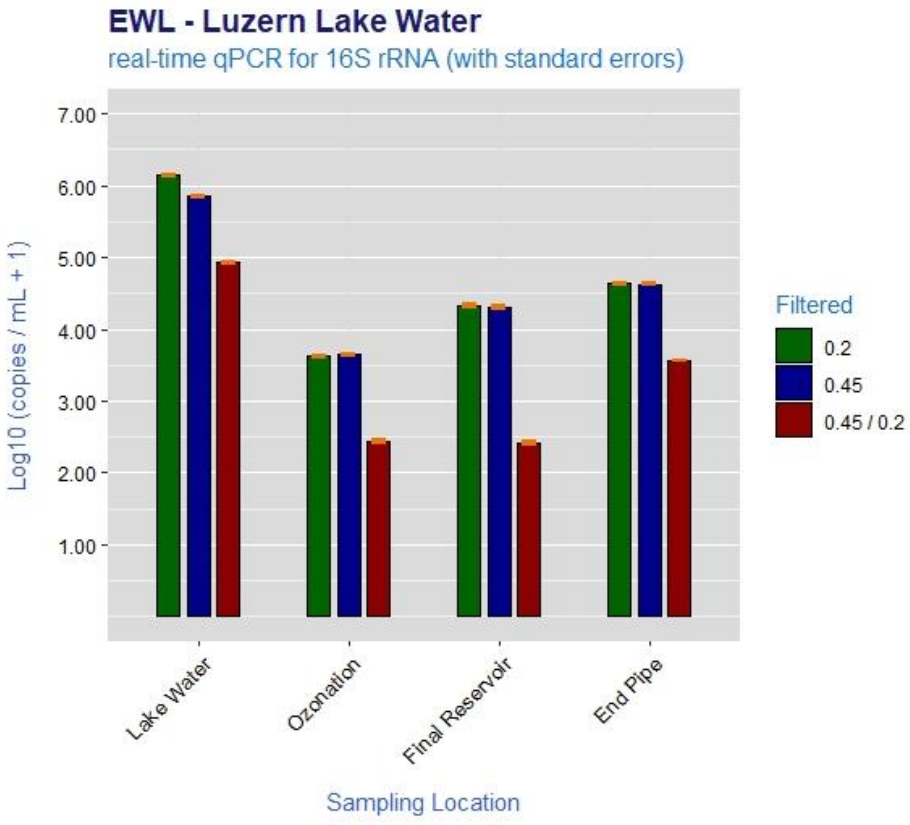
The high amount of three log10-values of *intI1* within HNA content bacteria from the end of pipe sample is mainly influenced by the DWTP using spring water (R-plot 8). There was no quantification or detection of *intI1* in bacteria with LNA content, except for the low amount of less than 10 copies/mL at the «End Pipe» location. The same can be seen in R-plot 9 for the ARG *sul1*, since there was no detection or quantification within LNA content bacteria, whereas *sul1* was detected in HNA content bacteria in the «End Pipe» sample with a relatively small amount of less than 5 copies/mL. However, within the plant using lake water *sul1* was not at quantifiable levels, it is not possible to say which plant was the origin of *sul1* in bacteria with HNA content at the «End Pipe» location. While with regards to *intI1* and *sul1* in the raw «Spring water» and the «End Pipe» sample, the DWTP using spring water even seems to have a negative effect, leading to an increase in HNA and LNA content bacteria (R-plot 8 and R-plot 9).

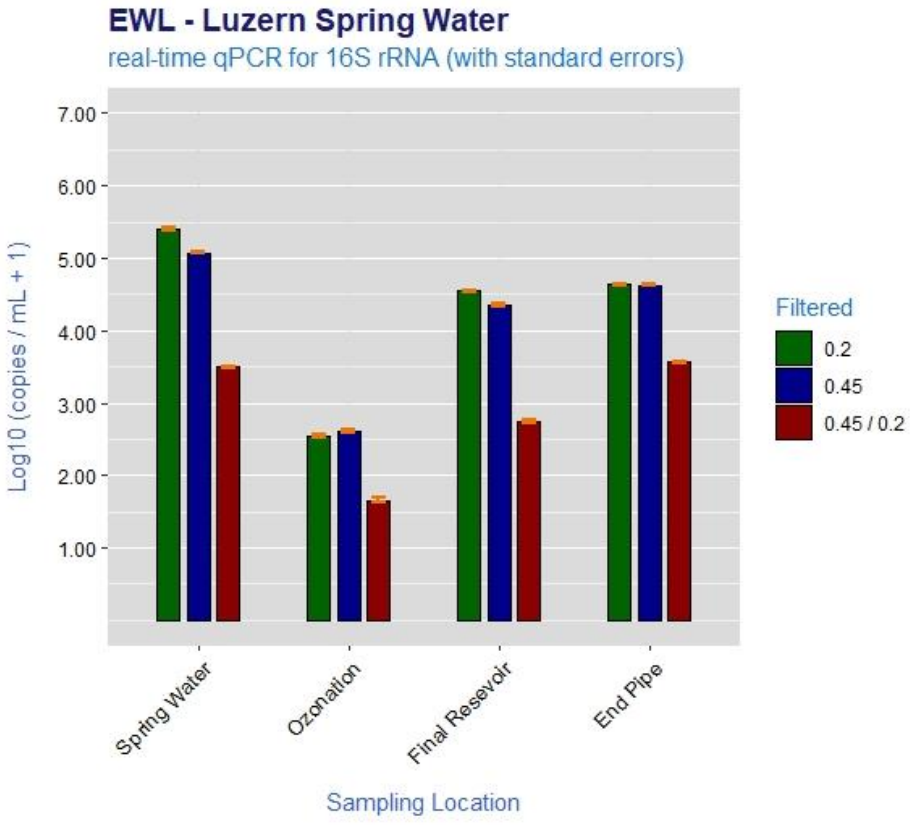


R-plot 5: FCM runs (SYBR Green I) for all sampling locations at the DWTP in Luzern using lake water.

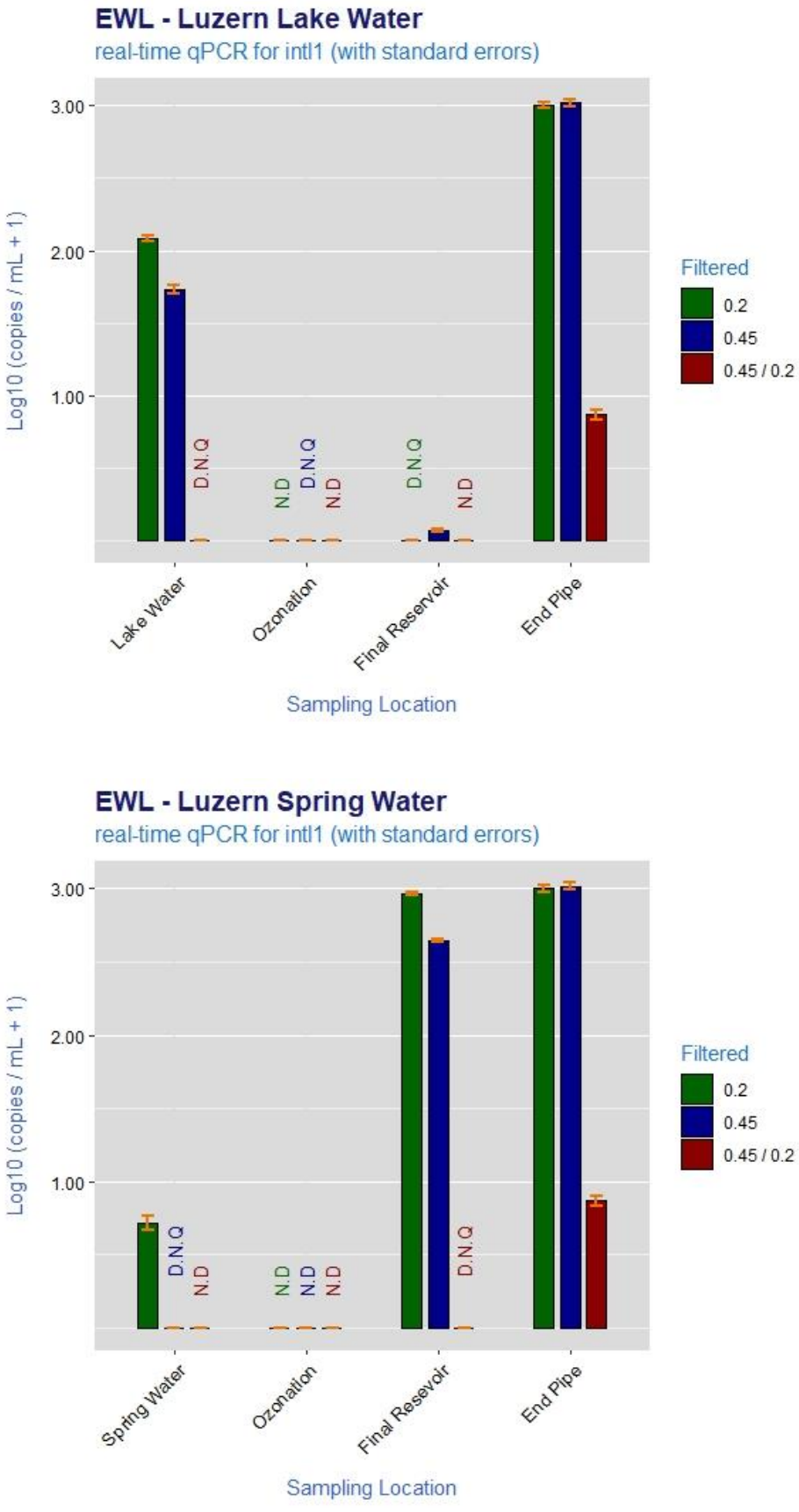


R-plot 6: FCM runs (SYBR Green I) for all sampling locations at the DWTP in Luzern using spring water.

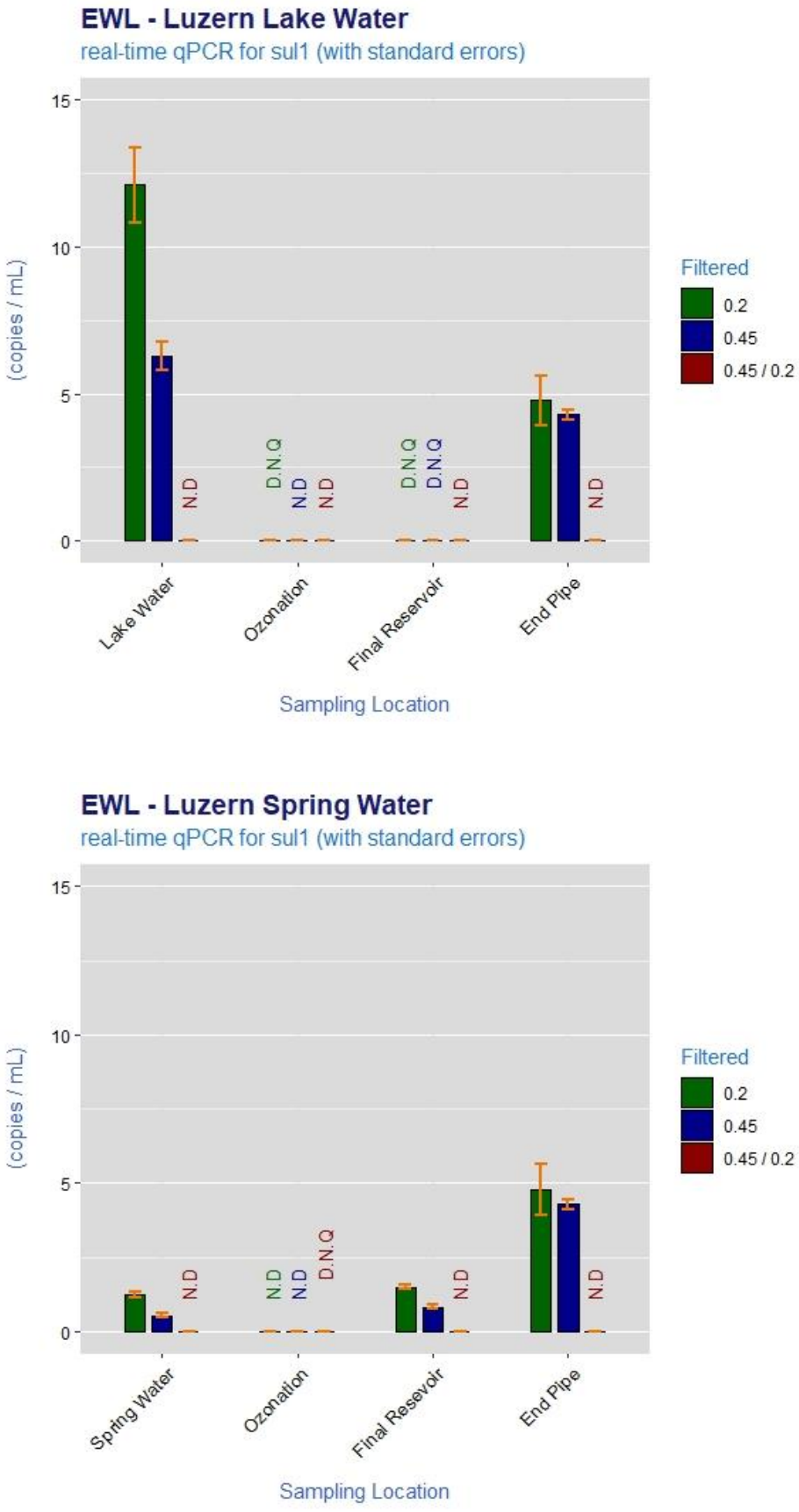




R-plot 7: qPCR for 16S rRNA for all sampling locations at the DWTPs in Luzern using lake and spring water.



R-plot 8: qPCR for *intI1* for all sampling locations at the DWTPs in Luzern using lake and spring water.



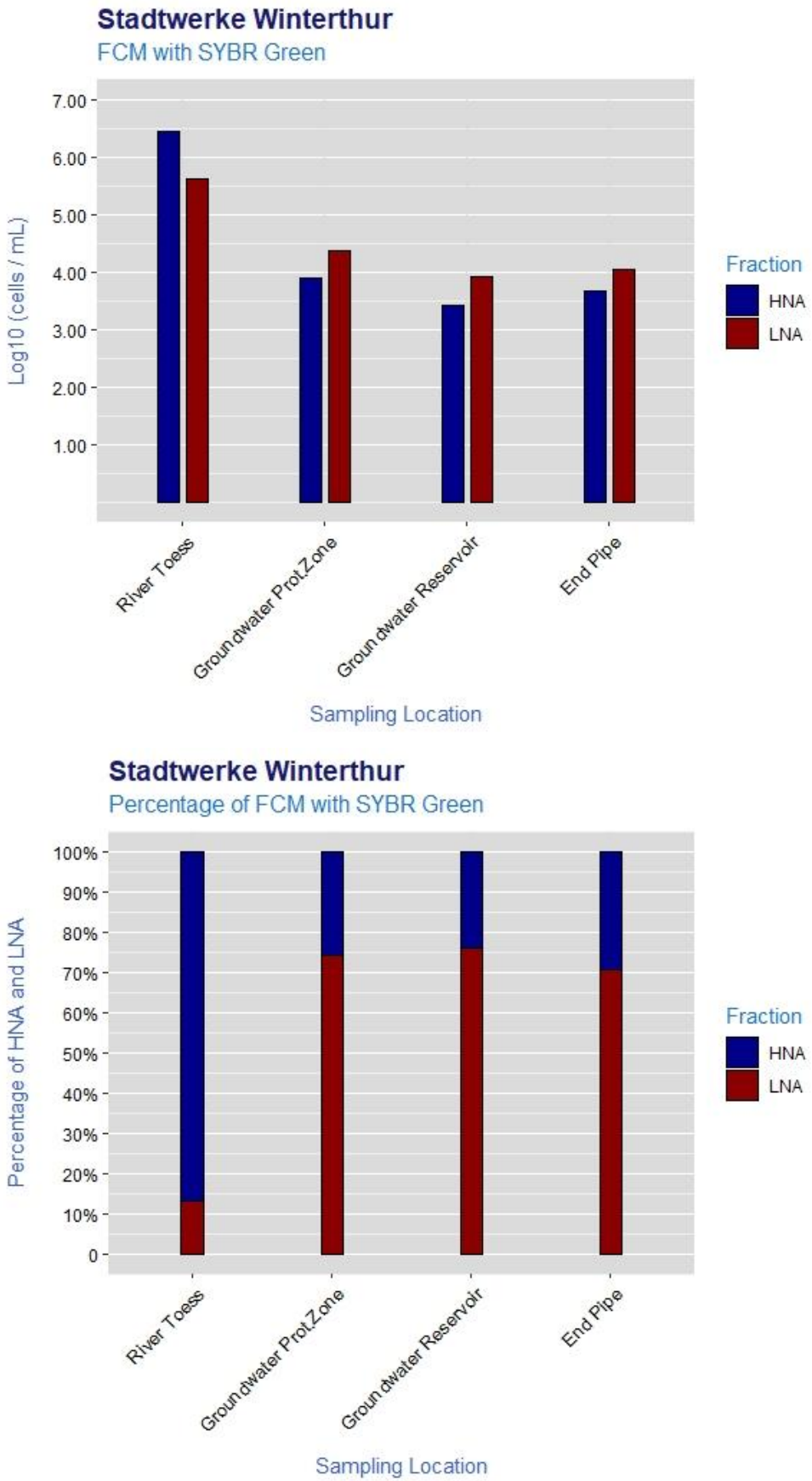
R-plot 9: qPCR for *sul1* for all sampling locations at the DWTPs in Luzern using lake and spring water.

Stadtwerke Winterthur

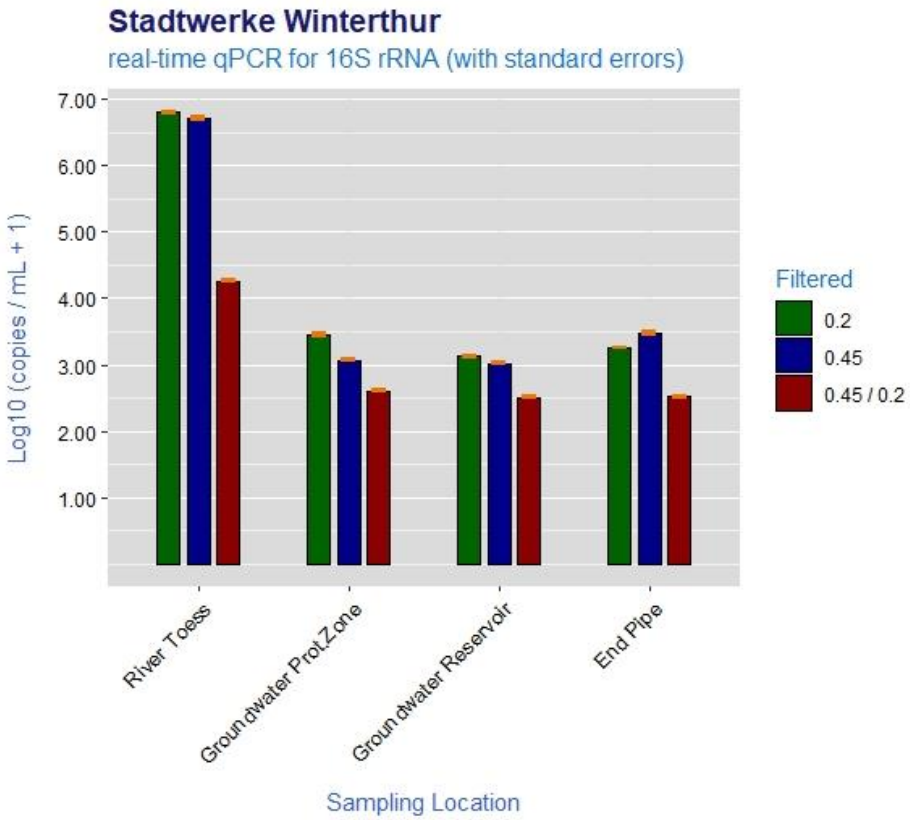
The river Töss had a high proportion of HNA (R-plot 10), whereas the groundwater infiltration, as observed in Basel (R-plot 1), had a higher effectiveness on HNA than on LNA cells. The percentage of HNA and LNA stayed inside the DWDS more or less on the same level.

The effectiveness of groundwater infiltration on bacteria with HNA content can be seen in R-plot 12, where the amount of 16S rRNA in HNA content bacteria decreased by a factor of $3x10^3$, while it went down by a factor of about 50 in bacteria with LNA content. Moreover, both groundwater sampling locations shared roughly the same amounts of HNA and LNA content bacteria. Although HNA content bacteria showed a slightly higher amount at the «End Pipe» location, a small increase inside the DWDS for bacteria with HNA content was observed.

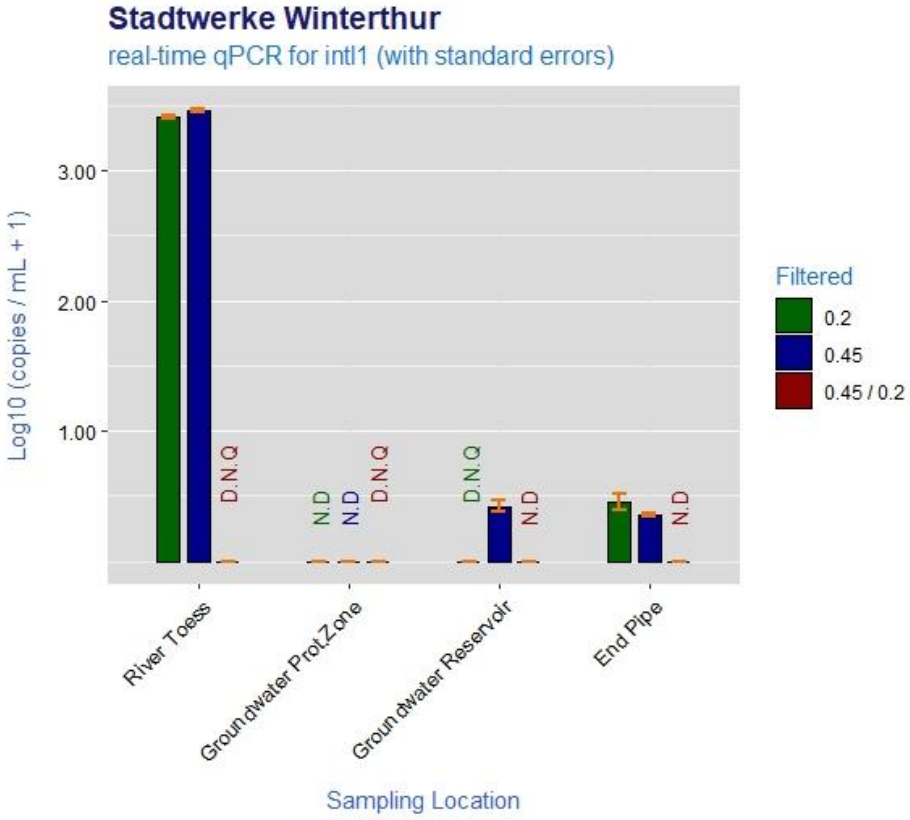
The *intI1* and *sul1* results in R-plot 11 and R-plot 13 shows that they could not be detected or quantified in bacteria with LNA content at all sampling locations. Additionally, one groundwater sample was not detected for HNA content bacteria from this DWTP. Nevertheless, the results showed that, at least for *intI1* in HNA content bacteria, no regrowth could be measured inside the distribution system. On the other hand, there could be a regrowth inside the distribution system in bacteria with HNA content carrying the ARG *sul1*, even if the amount is on a low level of about 10 copies/mL (R-plot 13).



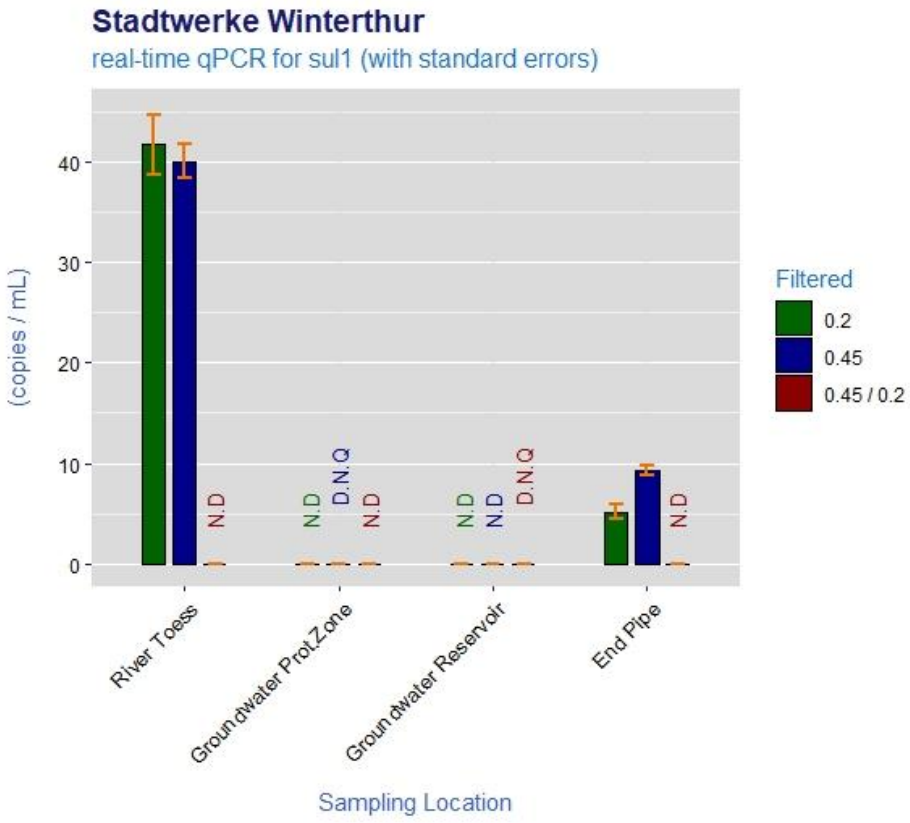
R-plot 10: FCM runs (SYBR Green I) for all sampling locations at the DWTP in Winterthur.



R-plot 12: qPCR for 16S RNA for all sampling locations at the DWTP in Winterthur.



R-plot 11: qPCR for *intI1* for all sampling locations at the DWTP in Winterthur.

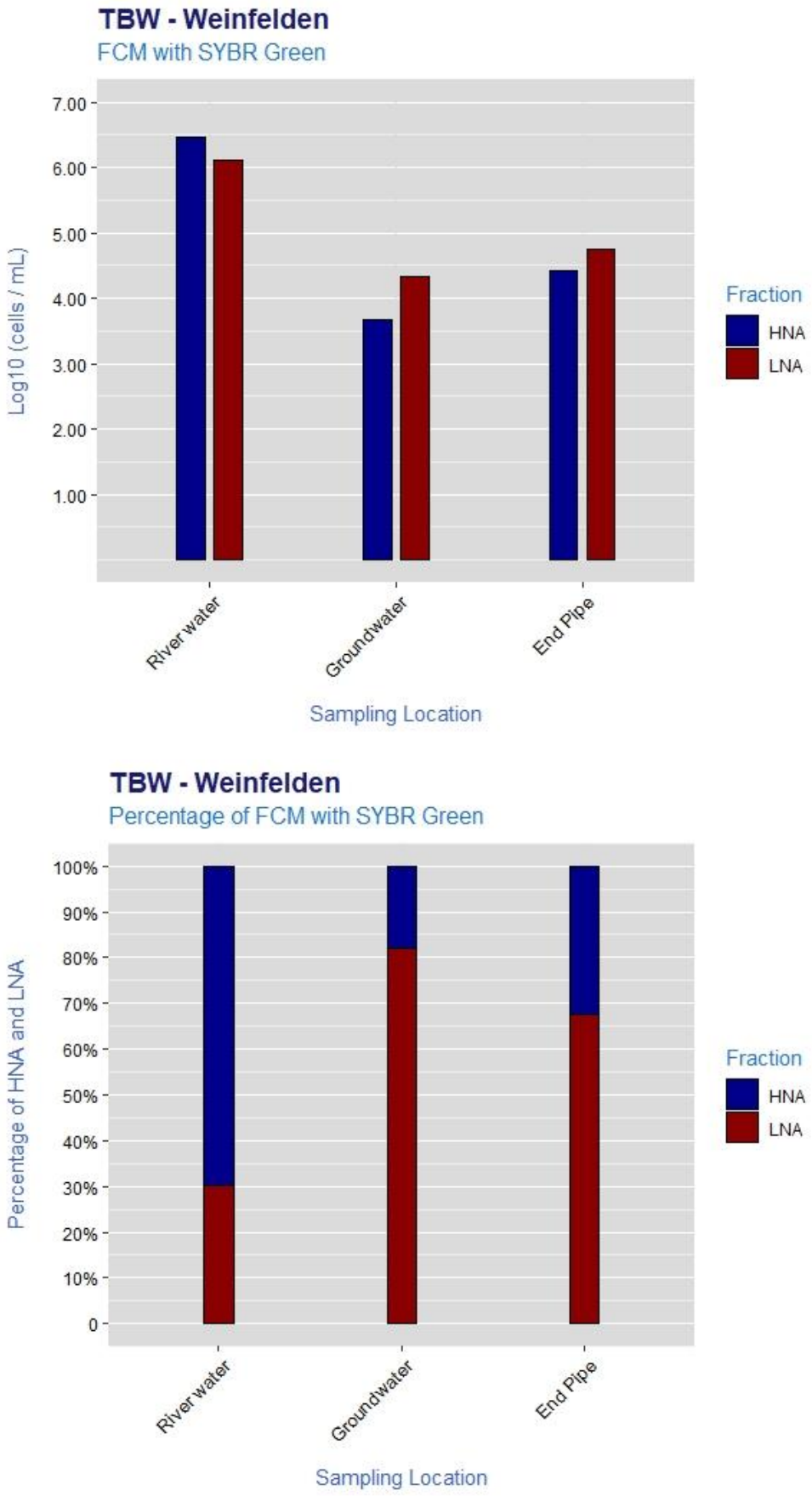


R-plot 13: qPCR for *sul1* for all sampling locations at the DWTP in Winterthur.

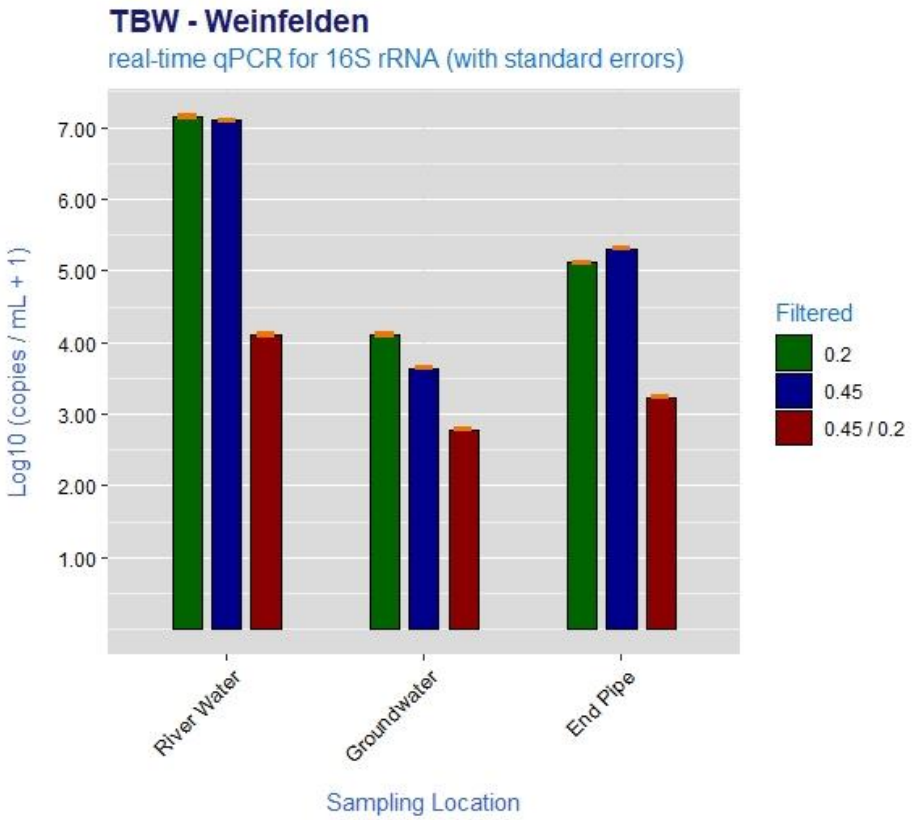
TBW - Weinfelden

The FCM results for the drinking water facility, led by «Technische Betriebe Weinfelden» (TBW) showed similar trends as the other samplings of river water that infiltrates into the groundwater (see R-plot 14). Showing that river water carried more HNA than LNA. Furthermore, infiltration into the groundwater has a lower effect on LNA cells and therefore the proportion of HNA cells is decreasing more between river water and groundwater. The graphics in R-plot 14 shows also, that both fractions increase inside the DWDS and that the proportion of HNA cells is rising by nearly 15%.

While the amount of bacteria in R-plot 16 is decreasing with infiltration into groundwater, there is a regrowth of HNA content bacteria inside the DWDS by a factor of nearly 50 and a slightly regrowth of LNA content bacteria by a factor of about three. The same regrowth is represented in R-plot 15 and R-plot 17 within HNA content bacteria of the real-time qPCRs for *intI1* and *sul1*. After decreasing with infiltration, there is a strong increase of *intI1* in bacteria with HNA content by two log10-values and a rather moderate increase of *sul1*, even though no *sul1* and just a small amount of approximately 2 copies/mL of *intI1* were detected in LNA content bacteria.

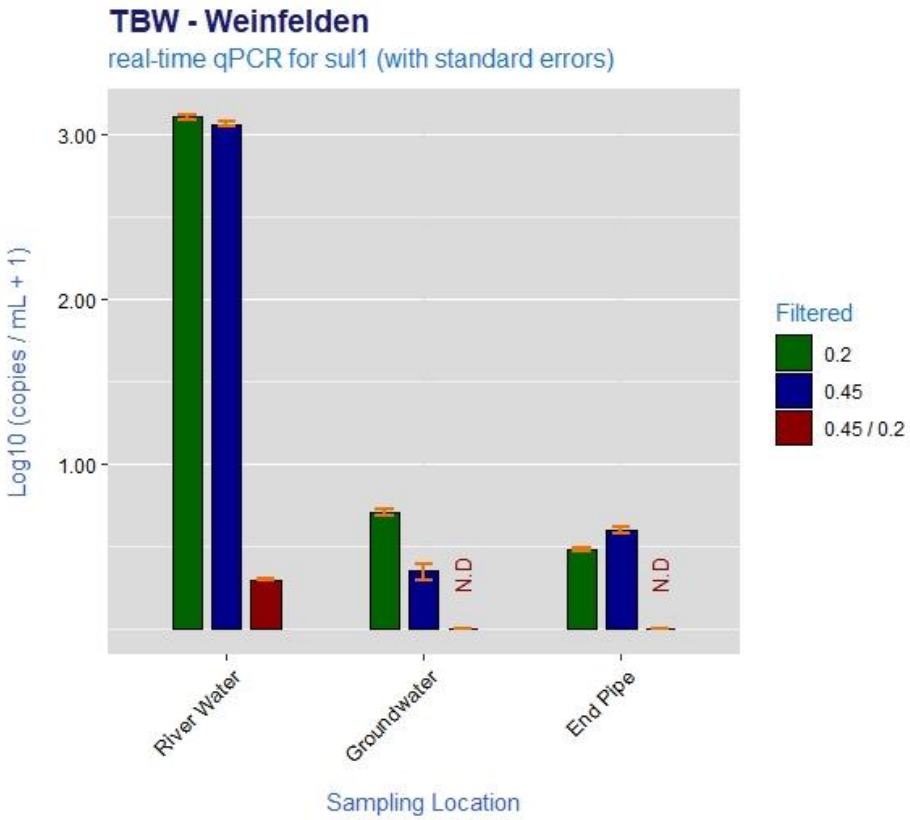


R-plot 14: FCM runs (SYBR Green I) for all sampling locations at the DWTP in Weinfelden.



R-plot 16: qPCR for 16S rRNA for all sampling locations at the DWTP in Weinfelden.

R-plot 15: qPCR for *intI1* for all sampling locations at the DWTP in Weinfelden.



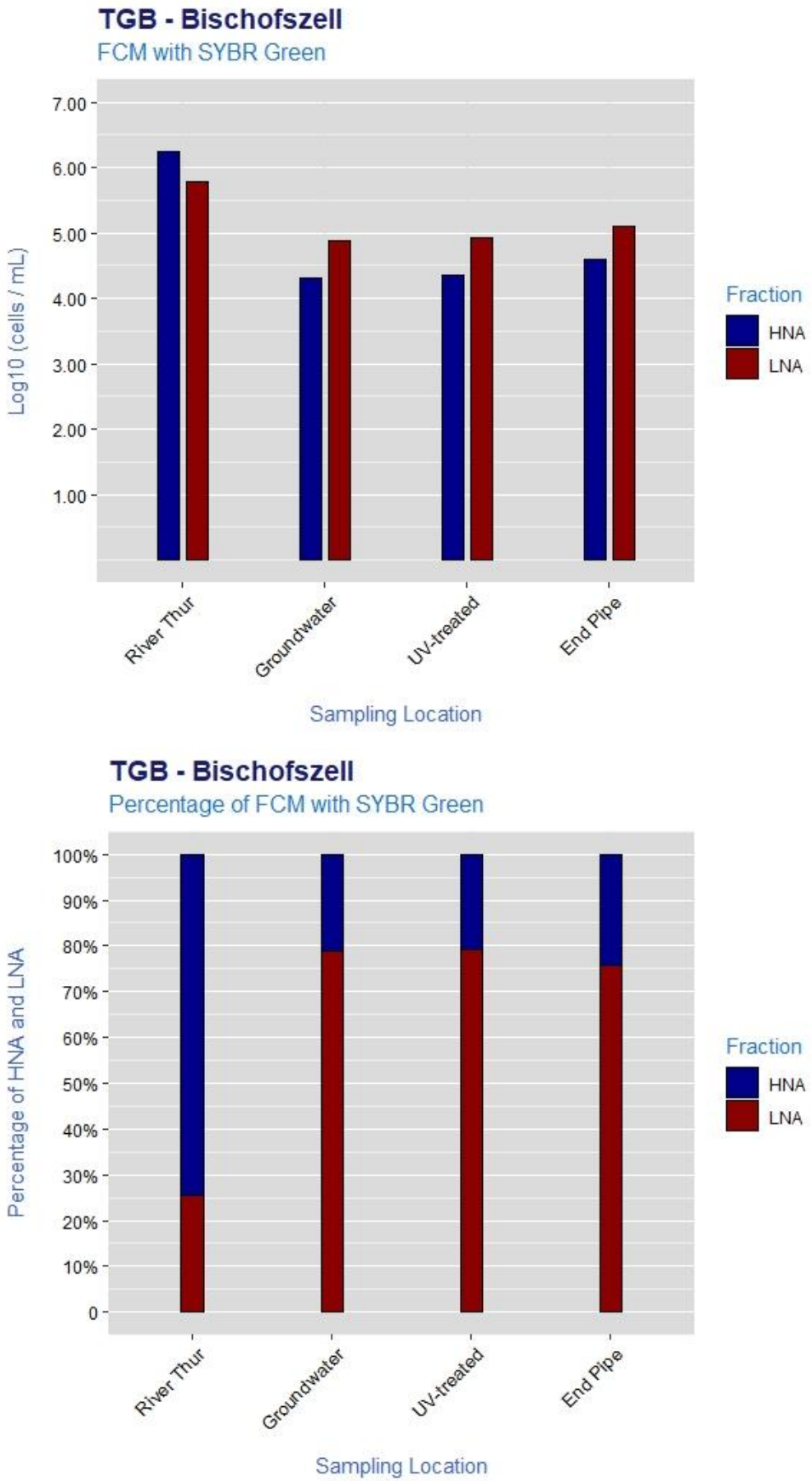
R-plot 17: qPCR for *sul1* for all sampling locations at the DWTP in Weinfelden.

TGB - Bischofszell

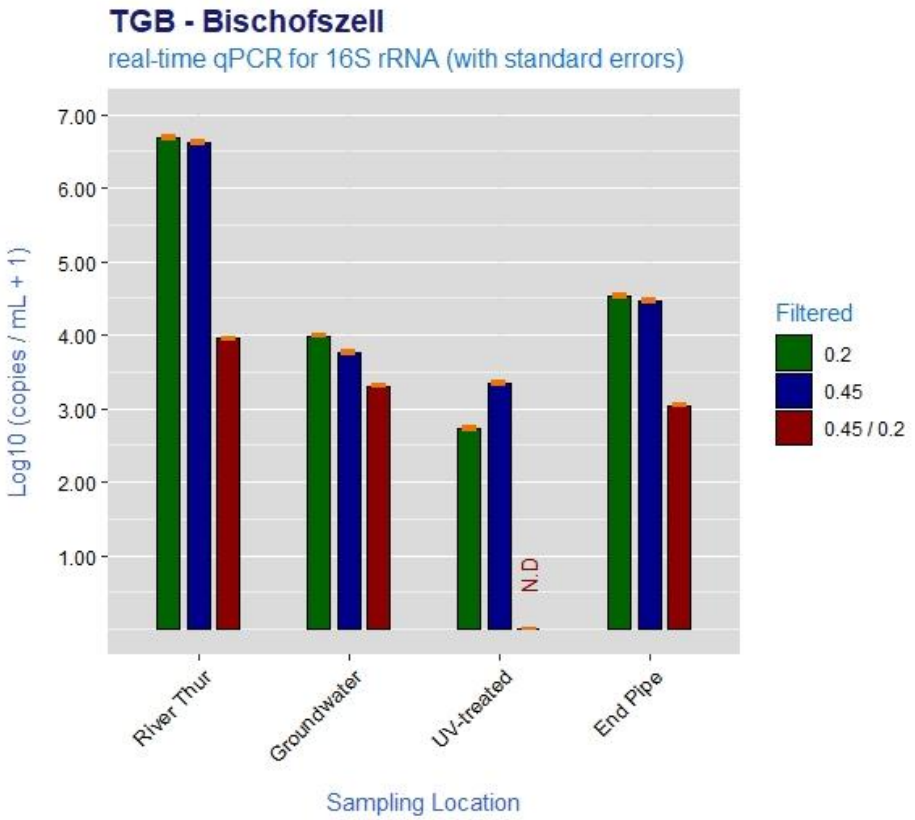
River Thur had comparable amounts of HNA and LNA cells measured with FCM as its sidearm river in Weinfelden downstream of Bischofszell (R-plot 14 and R-plot 18). R-plot 18 shows that groundwater infiltration has the same effect – bacteria with HNA content decreases more strongly than LNA content bacteria - as was discovered in Weinfelden, Winterthur or Basel (R-plot 1, R-plot 10 and R-plot 14). Moreover, no effect of regrowth was measured in the drinking water system in Bischofszell, led by «Technische Gemeindebetriebe» (TGB), between the «UV-treated» location (start of the DWDS) and «End Pipe» sample.

Results from real-time qPCR for 16S rRNA shows a decrease in bacteria with HNA content, by a factor of $7x10^2$, whereas LNA content bacteria decreased by a factor of nearly four (R-plot 20). Even though it was not possible to detect bacteria with LNA content in the «UV-treated» sample, HNA content bacteria increased inside the distribution, by a factor of more or less ten.

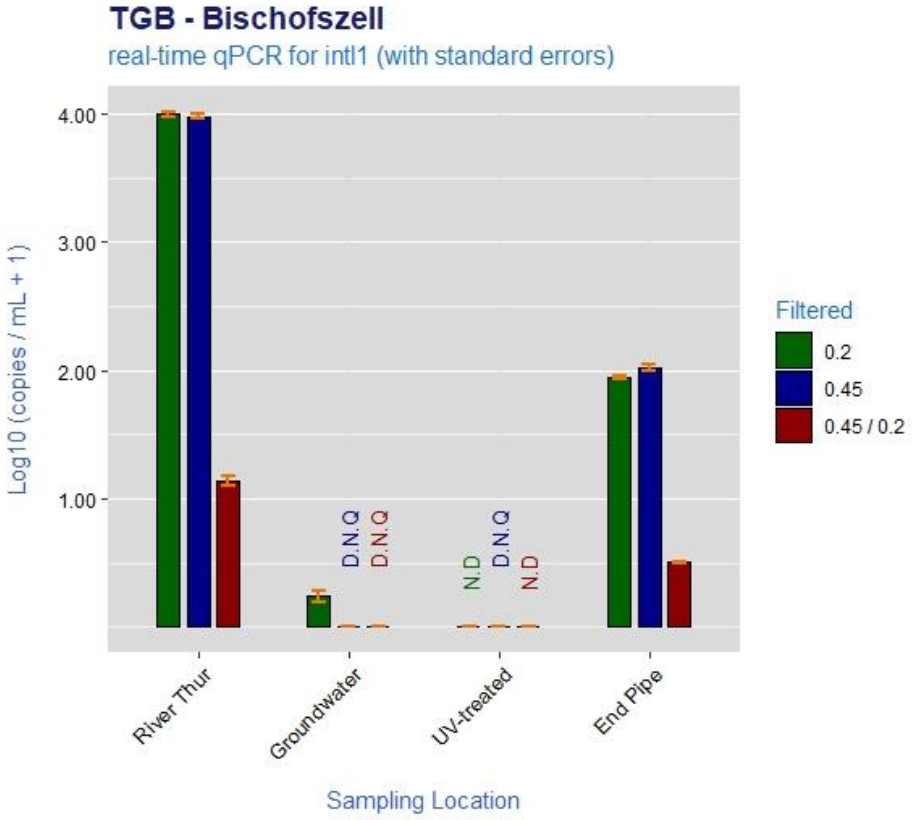
The ARG *sul1* and the class1 integron-integrase gene *intI1* were not detected or were detected but not quantifiable in the «Groundwater» and «UV-treated» samples, while it was possible to detect *sul1* in HNA content bacteria at the end of pipe location with a small amount of about 2 copies/mL (R-plot 21). The *intI1* gene was detected in HNA as in LNA content bacteria in the end of pipe location, while it was impossible to identify the source of the target gene-harbouring organisms in the DWDS due to the mostly unquantifiable measurements (R-plot 19).



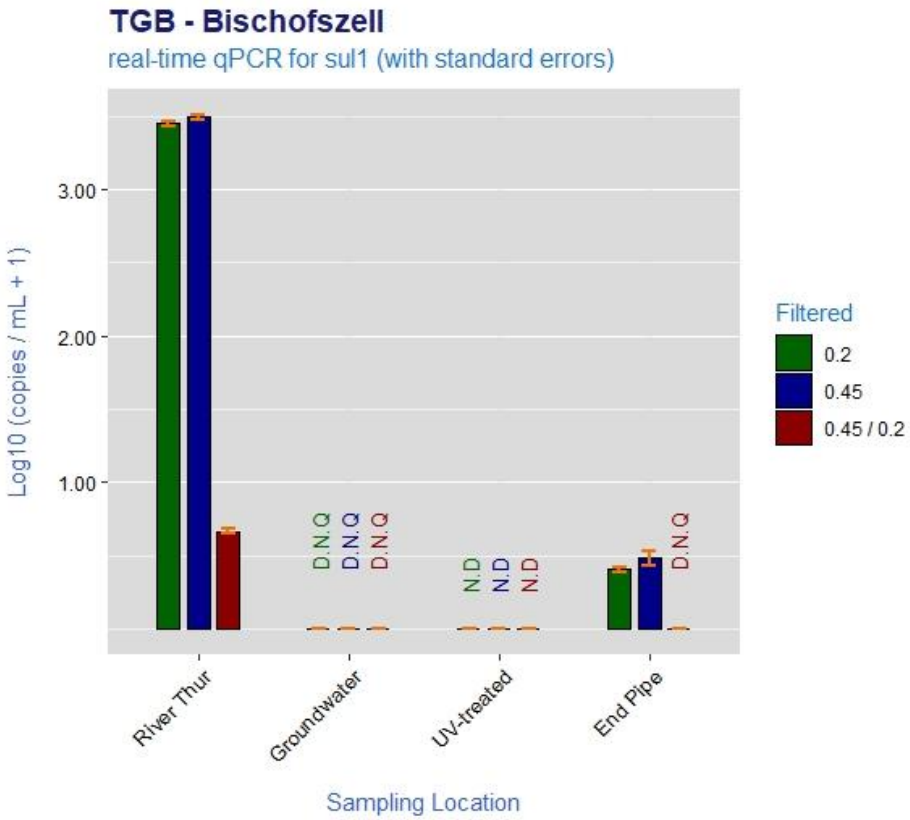
R-plot 18: FCM runs (SYBR Green I) for all sampling locations at the DWTP in Bischofszell.



R-plot 20: qPCR for 16S rRNA for all sampling locations at the DWTP in Bischofszell.



R-plot 19: qPCR for *intI1* for all sampling locations at the DWTP in Bischofszell.



R-plot 21: qPCR for *sul1* for all sampling locations at the DWTP in Bischofszell.

3.3 Analysis of HNA and LNA

The relative abundances of HNA and LNA in comparison to the 16S rRNA were calculated for qPCR samples with quantifiable values, as it was not possible to calculate the D.N.Q or N.D values (Table 4). In this table, the proportion of HNA and LNA is given for all samples where the relative abundance for both fractions could be calculated. For samples classified as D.N.Q, the average copies/mL of the LOQs were used to get an idea of the relative abundances. To calculate the relative abundance, the total average copies/mL of HNA or LNA (0.45 µm or 0.45/0.2 µm filtered) were divided by the total average copies/mL of 16S rRNA. Table 4 shows that the relative abundances of the ARG *sul1* were lower than for *intI1*, except for HNA in the «Winterthur – End Pipe» sample. Moreover, the relative abundances were lower in LNA than HNA in all samples.

Table 4: Relative abundance of *intI1* and *sul1* in comparison to the 16S rRNA from real-time qPCR. The average copies/mL of the LOQs were used for values classified as D.N.Q.

Relative abundance in comparison to the 16S rRNA	***intI1***		***sul1***	
Sample Location	**HNA**	**LNA**	**HNA**	**LNA**
IWB – River Rhein	6.20E-04	2.12E-04	5.11E-05	2.23E-05
IWB – End Pipe	2.36E-04	(LOQ) < 3.31E-05	1.64E-05	
EWL – Lake Water – Raw Lake Water	7.57E-05	(LOQ) < 3.56E-07	8.91E-06	
EWL – Spring Water - Final Reservoir	1.99E-02	(LOQ) < 5.51E-05	3.72E-05	
EWL – End Pipe	2.47E-02	1.75E-03	1.02E-04	
Winterthur – River Töss	5.55E-04	(LOQ) < 1.66E-06	7.71E-06	
Winterthur – End Pipe	4.17E-04		3.07E-03	
TBW – River water	1.35E-03	2.85E-04	9.27E-05	7.66E-05
TBW – End Pipe	9.51E-03	9.19E-04	1.46E-05	
TGB – River Thur	2.25E-03	1.43E-03	7.33E-04	4.10E-04
TGB – End Pipe	3.56E-03	1.95E-03	7.02E-05	(LOQ) < 2.07E-05

Furthermore, the calculated proportions of relative abundances in comparison to the 16S rRNA for all samples were higher for HNA than for LNA (Table 5). For *intI1*, the proportions of HNA were even very high (more than 80%) in some samples. The table also shows that the relative abundance of HNA in the end of pipe samples was higher than in the raw water samples for TBW (Weinfelden) and TGB (Bischofszell), which could indicate a concentration of *intI1* in the DWTPs or DWDSs.

Table 5: Proportion of the relative abundance in comparison to the 16S rRNA from real-time qPCR. The average copies/mL of the LOQs were used for values classified as D.N.Q.

Proportion of relative abundance in comparison to the 16S rRNA	***intI1***		***sul1***	
Sample Location	**HNA (%)**	**LNA (%)**	**HNA (%)**	**LNA (%)**
IWB – River Rhein	74.54	25.46	69.66	30.34
IWB – End Pipe	87.68	(LOQ) < 12.32		
EWL – Lake Water – Raw Lake Water	99.53	(LOQ) < 0.47		
EWL – Spring water - Final Reservoir	99.72	(LOQ) < 0.28		
EWL – End Pipe	93.39	6.61		
Winterthur – River Töss	99.70	(LOQ) < 0.30		
TBW – River water	82.61	17.39	54.71	45.29
TBW – End Pipe	91.19	8.81		
TGB – River Thur	61.09	38.91	64.13	35.87
TGB – End Pipe	64.56	35.44	77.21	(LOQ) < 22.79

The gene copies per cell were calculated for the same samples as in Table 4, dividing the real-time qPCR results in average copies/mL by the total amount of cells/mL within HNA or LNA from the FCM results. In this table, the results showed that in bacteria with HNA content are usually more copies of the genes within the cells than in LNA content bacteria (Table 6). As this shows that the 16S rRNA has more gene copies per cell in HNA content bacteria, the relative frequency of carrying an ARG is overestimated compared to the 16S rRNA in Table 4 and its proportion in Table 5 for LNA content bacteria.

Table 6: Gene copies per cell in HNA and LNA. The average copies/mL of the LOQs from real-time qPCR were used for values classified as D.N.Q to calculate the gene copies per cell.

Gene copies per cell in HNA and LNA	**16S rRNA**		***intI1***		***sul1***	
Sample Location	**HNA**	**LNA**	**HNA**	**LNA**	**HNA**	**LNA**
IWB – River Rhein	1.8E+00	9.2E-02	1.1E-03	2.0E-05	9.2E-05	2.1E-06
IWB – End Pipe	1.7E-01	5.6E-02	4.0E-05	1.8E-06	2.8E-06	
EWL – Lake Water – Raw Lake Water	1.3E+00	7.7E-01	9.6E-05	(LOQ) < 2.7E-07	1.1E-05	
EWL – Spring Water - Final Reservoir	2.4E-01	3.5E-02	4.8E-03	(LOQ) < 1.9E-06	7.6E-07	
EWL – End Pipe	2.7E-01	2.0E-01	6.8E-03	3.5E-04	2.8E-05	
Winterthur – River Töss	1.7E+00	3.9E-01	9.4E-04	(LOQ) < 6.5E-07	1.3E-05	
Winterthur – End Pipe	2.6E-01	7.7E-02	1.1E-04		7.9E-04	
TBW – River water	3.2E+00	4.2E-02	4.3E-03	1.2E-05	2.9E-04	3.3E-06
TBW – End Pipe	2.8E+00	1.5E-01	2.7E-02	1.4E-04	4.1E-05	
TGB – River Thur	1.9E+00	4.4E-02	4.3E-03	6.4E-05	1.4E-03	1.8E-05
TGB – End Pipe	2.0E-01	6.5E-02	7.1E-04	1.3E-04	1.4E-05	(LOQ) < 1.3E-06

Compared to the relative frequency in 16S rRNA in Table 5, the proportion of gene copies per cell in bacteria with HNA and LNA content (Table 7) shows even higher proportions in HNA content bacteria. Therefore, bacteria with LNA content are even less capable of carrying an ARG as shown in Table 5.

Table 7: Proportion of gene copies per cell in HNA and LNA. The average copies/mL of the LOQs from real-time qPCR were used for values classified as D.N.Q to calculate the gene copies per cell.

Proportion of gene copies per cell in HNA and LNA	**16S rRNA**		***intI1***		***sul1***	
Sample Location	**HNA (%)**	**LNA (%)**	**HNA (%)**	**LNA (%)**	**HNA (%)**	**LNA (%)**
IWB – River Rhein	95.11	4.89	98.28	1.72	97.81	2.19
IWB – End Pipe	75.47	24.53	95.64	(LOQ) < 4.36		
EWL – Lake Water – Raw Lake Water	62.35	37.65	99.72	(LOQ) < 0.28		
EWL – Spring Water - Final Reservoir	87.29	12.71	99.96	(LOQ) 0.04		
EWL – End Pipe	57.72	42.28	95.07	4.93		
Winterthur – River Töss	81.11	18.89	99.93	(LOQ) < 0.07		
TBW – River water	98.69	1.31	99.72	0.28	98.91	1.09
TBW – End Pipe	94.97	5.03	99.49	0.51		
TGB – River Thur	97.73	2.27	98.54	1.46	98.72	1.28
TGB – End Pipe	75.41	24.59	84.88	15.12	91.23	(LOQ) < 8.77

The number of samples that were classified as D.N.Q or N.D in all HNA and LNA samples (0.2 µm filtered excluded) of the real-time qPCR results are summarized in Table 8. It shows that 21.73% of all samples could not be detected and 15.93% of them were unquantifiable, whereas the most of the N.D and D.N.Q samples appeared in groundwater or samples from treated water inside the DWTPs. Half of the samples from ozonation and about 40% of the UV-treatment could not be detected, while approximately 36% of all groundwater samples were not quantifiable and 20% not detected. Additionally, more samples of LNA were not detected (80% LNA to 20% HNA classified as N.D), while more or less equal amounts of samples with HNA and LNA content were not quantifiable.

Table 8: Number of samples that were detected but not quantifiable (D.N.Q) or not detected (N.D) of all real-time qPCR results for HNA and LNA (0.2 µm filtered samples excluded).

Number of D.N.Q and N.D in samples		**D.N.Q**		**N.D**	
Sample Location	**Samples**	**HNA**	**LNA**	**HNA**	**LNA**
River water	24				2
Lake water	6		1		1
Spring water	6	1			2
Groundwater	30	5	6	2	4
Ozonation	12	1	1	3	3
UV-treated	12	2	1	1	4
Final Reservoir	12	1	1		3
End Pipe	36		2		5
Total	138	10	12	6	24
Total percent	100%	7.24%	8.69%	4.34%	17.39%
		15.93%		21.73%	
Proportion in HNA and LNA		45.45%	54.55%	20.00%	80.00%

Compared to the number of samples of real-time qPCR for *intI1* and the ARG *sul1* (Table 9), the ratio shows that more samples for *intI1* in LNA content bacteria were undetectable or not quantifiable, while slightly more samples were unquantifiable for *sul1* in HNA content bacteria, but four times more in bacteria with LNA content could not be detected. In addition, more samples in both fractions for *intI1* were classified as D.N.Q than N.D, whereas in *sul1* more samples were classified as N.D.

Table 9: Number of samples for *intI1* and *sul1* that were detected but not quantifiable (D.N.Q) or not detected (N.D) of all real-time qPCR results for HNA and LNA (0.2 µm filtered samples excluded).

Number of D.N.Q and N.D in target genes		**D.N.Q**		**N.D**	
Target genes	**Samples**	**HNA**	**LNA**	**HNA**	**LNA**
intI1	46	5	9	2	7
	100%	10.86%	19.56%	4.34%	15.21%
sul1	46	5	4	4	16
	100%	10.87%	8.69%	8.69%	34.78%

4 Discussion and outlook

Interestingly, in this study, when testing water samples from six different DWTPs in Switzerland, the ARG *sul1* was never detected with real-time qPCR in all treated samples, while detection occurred in river water samples with higher HNA content bacteria copies/mL (Basel, Weinfelden, Bischofszell). The question though arises, how precise the filtration on 0.45 µm and 0.2 µm filter membranes separates HNA and LNA content bacteria. It has already been observed by Proctor et al. (2018), that this filtration method separates «small» LNA content bacteria from large bacteria, separating them by cell size rather than genome size (Proctor et al., 2018). Taking into account the different shapes of the bacteria - whereas it was already found that 10% of the microbial community were able to pass through 0.2 µm membranes (Wang et al., 2009) - it is possible that bacteria with for example thin and long shapes could pass through the 0.45 µm membrane but still belong to the bacteria with HNA content or vice versa. Therefore, when using this filter method, bacteria with LNA content should be classified as «small» LNA content bacteria.
In comparison, it seems that this filter issue - which is even more a definition problem of HNA and LNA content bacteria -took place when the HNA amount in qPCR for *sul1* was higher than 100 copies/mL. According to this, it is not certain that there is a minimum genome size for carrying ARGs, but the results nevertheless showed a potential for this possibility. For a more detailed investigation, the bacteria in the river water samples with «small» LNA content, which carries the ARG *sul1*, could be analysed in a phylogenetic study.

Furthermore, the FCM results showed, as revealed in previous studies by Proctor et al. (2018) and Van Nevel et al. (2017), that the FCM measurement was a fast method to detect and separate HNA and LNA cells in water samples and will be a practical method in further drinking water studies to investigate the treatment effectiveness on these fractions in DWTPs. The FCM measurement revealed a better removal of HNA than LNA in DWTPs, especially due to filtration steps, which makes sense since HNA content bacteria have in general bigger cell bodies. Therefore, the LNA content bacteria have a higher chance to pass through current DWTP processes and could be transported to the final consumer, which is an important reason for studying ARGs in LNA content bacteria. However, there is one major disadvantage of the FCM method in DWTPs when it comes to UV-disinfection. Because UV irradiation has no effect to the cell membrane but direct damage to the plasmid with the ARG (Sharma et al., 2016), the FCM method cannot be used for UV-disinfection. The results of FCM will not reveal any effect on UV-disinfection, which may give a false impression of the effectiveness of this treatment step (R-plot 18). To see whether UV irradiation works (R-plot 20), HPC plate count would be the more accurate option.

Additionally, the results of real-time qPCR for 16S rRNA and FCM with SYBR® Green I measuring total cells/mL, revealed quite similar amounts of bacteria with HNA and LNA content in all «End Pipe» samples, while in Winterthur the amounts were one log10-value lower. The differences in the «End Pipe» sample in Winterthur shows, that the steps of drinking water treatment, especially in the case of ARGs, also depend on the water source. In Winterthur, water from an alpine river is used, which is, compared to other rivers like the river Rhein in Basel or the river Thur in Weinfelden and Bischofszell, less influenced by natural causes or by humans due to effluents from wastewater treatment plants or other sources of ARGs, such as agricultural land use and livestock farms. It is therefore recommended that DWTPs, using water sources that are more exposed to such impacts from ARGs, take this into account in their treatment steps. However, in comparison to the study of Blanc & Schädler (2014), which showed that drinking water in Switzerland has the level of quality that can compete with that of mineral water, all tested DWTPs seem to deliver water with this level of quality to the end users (Blanc & Schädler, 2014).

Although, the regrowth of bacteria of one log10-value for HNA and a slightly regrowth of LNA could be observed due to FCM total cell counting in the distribution system of Weinfelden (R-plot 14), while an increase also could be observed in real-time qPCR for 16S rRNA and *intI1* (R-plot 16 and R-plot 15). Since Engemann et al. (2008) found out that tetracycline resistance genes migrated into biofilms, they suggested biofilms as potential long-term reservoirs for ARGs (Engemann et al., 2008). In addition, Petrovich et al. (2019) found in their study of the abundances of different ARGs in biofilms – targeting *sul1*, *qnrS*, *ermB* and the class1 integron-integrase gene *intI1* – that cells on the surface of biofilms tend to have *intI1* more frequently (Petrovich et al., 2019). Therefore, it might be useful for the DWTP in Weinfelden to consider general disinfection in order to remove potential biofilms within the distribution system, by either using chlorine, chloramines or cleaning via flushing or pigging, which are considered by Liu et al. (2016) to be the best routine management practices for biofilm control (Liu et al., 2016). Furthermore, it is assumed that chloramines can penetrate biofilms better than chlorine (Liu et al., 2016), so the use of chloramines would be recommended if chlorine could not bring the hoped-for results. Besides, a study of Hwang et al. (2012) investigated the effects of chlorination and chloramination in a DWDS in Urbana (Illinois, U.S.A) over 2 years and revealed that the reversible shifts in microbial communities were especially pronounced with chloramination (Hwang et al., 2012). Moreover, according to Hwang et al. (2012), many water facilities seem to switch from using chlorine for disinfection to chloramines due to the rising concern of disinfection by-products formed during chlorination (Hwang et al., 2012).

The gene copies per cell, in samples where it was possible to compare both fractions (Table 6) and their proportions (Table 7), showed higher frequencies of *intI1* and *sul1* in HNA than «small» LNA content bacteria. In general, except in the «TGB – End Pipe» sample from Bischofszell, the proportions in «small» LNA content bacteria were below 5%. Therefore, with regard to ARGs in the drinking water sector, it might be useful to focus more on the reduction of HNA instead of LNA content bacteria. The most effective treatment step to reduce bacteria with HNA content was identified by infiltration into groundwater, while another strong effect was observed by ozonation in both DWTPs in Luzern (R-plot 7). Stange et al. (2019) researched for the removal of antibiotic resistance genes during chlorination, ozonation and UV-disinfection and also found that ozonation has a strong effect (Stange et al., 2019). Nonetheless, the decrease in Luzern is followed by an increase after ozonation inside the DWTPs, especially in the DWTP using spring water. However, it must be emphasised that spring water already had more or less the same amounts of 16S rRNA copies/mL from qPCR and cells/mL from FCM as other end of pipe samples from Basel, Weinfelden or Bischofszell. Thus, the number of cells is unlikely to have a critical impact on the end users around Luzern, although special care to this increase after ozonation might still be required for the DWTP using spring water.

Finally, a potential larger risk due to ARGs in DWTPs with less or without treatment was not detected, while the infiltration of river water into the groundwater showed a high effectiveness in the reduction of the class 1 integron-integrase gene *intI1*, the ARG *sul1* and the total amount of bacteria, especially HNA (3.2). This effectiveness in reducing *intI1* and *sul1* due to infiltration may be related to previous studies that investigated the removal of ARGs in constructed wetlands (Lamori et al., 2019; Yi et al., 2017). The study of Lamori et al. (2019) showed overall reduction rates for *intI1* by 67.2% and for the ARG *erm(F)* by 13.1% in a constructed wetland, whereas Yi et al. (2017) found in their study targeting sixteen ARGs – including four β-lactam (*blaNDM1*, *blaKPC*, *blaCTX*, *blaSHV*), three sulfonamide (*sul1*, *sul2*, *dfrA1*), two macrolide (*qnrA*, *qnrB*), two tetracycline (*tetM*, *tetO*), one aminoglycoside (*aac6*) and one vancomycin (*vanA*) resistance genes – good removal for three types of ARGs (*intI1*, *sul1*, *sul2)*, which were significantly eliminated with removal efficiencies of over 90% in a full-scale constructed wetland system. Moreover, another study of Böckelmann et al. (2009) investigated six ARGs – ampicillin resistance (*ampC*), erythromycin resistance (*ermB*), methicillin resistance (*mecA*), extended lactam resistance (*blaSHV-5*), tetracycline resistance (*tetO*) and vancomycin resistance (*vanA*) - in three artificial aquifer recharge systems, one in Sabadell (Spain), one in Nardò (Italy) and one in Torreele (Belgium) and detected only *tetO* in groundwater samples of two systems (Böckelmann et al., 2009).

However, the removal of ARGs with infiltration of surface water into groundwater has not yet been widely studied. Therefore, a potential larger risk of ARGs in wastewater treatment plants with less or no treatment, using groundwater that is affected by surface water infiltration, could be better investigated in further studies aiming at a wider variation of ARGs, for instance the tetracycline resistance gene *tet0*, which was already found in groundwater by Böckelmann et al. (2009). Since this study has shown that LNA content bacteria have a higher chance than HNA content bacteria to undergo DWTP processes, the focus should also be on a broader variation of ARGs in LNA cells. Nevertheless, the amounts of ARGs in all end of pipe samples in this study were not dramatically high, as most of them were unquantifiable or were not detected.

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Leu Thomas	(EWL, Luzern)
Matuszkiewicz Joanna	(Stadtwerk Winterthur)
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Jularic Ante	(TGB, Bischofszell)

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List of figures, tables and R-plots

Annex

Annex 1. Information to sample locations

1.	Basel

Sampling date	Nearest weather station information		
08.06.2020	Basel	BAS	610911 / 265603
	Precipitation during sampling		0.0 mm
	Precipitation 24h before sampling		0.0 mm

Number	Location	Filter (µm)	Filtered amount duplication 1 + duplication 2 (L)
1.1	River Rhein	0.2	1.5 + 1.5
		0.45	1.5 + 1.5
		0.45/0.2	5 + 5
1.2	Groundwater	0.2	5 + 5
		0.45	9 + 9
		0.45/0.2	9 + 9
1.3	UV-treated	0.2	5 + 2.5
		0.45	10 + 8
		0.45/0.2	10 + 7.5
1.4	End Pipe	0.2	4 + 2.5
		0.45	10 + 8
		0.45/0.2	10 + 8

Treatment Steps
a) Rapid sand filtration b) Groundwater infiltration c) Activated charcoal filtration d) UV-disinfection

Retention time in DWDS until End Pipe	2 – 3 days

Weather data obtained from: https://gate.meteoswiss.ch/idaweb;
obtained weather data period - 01.06.2020 - 15.07.2020

2. Luzern Lake Water

Sampling date	Nearest weather station information		
23.06.2020	Luzern	LUZ	665539 / 209847
	Precipitation during sampling		0.0 mm
	Precipitation 24h before sampling		0.0 mm

Number	Location	Filter (µm)	Filtered amount duplication 1 + duplication 2 (L)
2.1	Lake Water (Vierwaldstättersee)	0.2	2 + 2
		0.45	4 + 4
		0.45/0.2	5 + 5
2.2	Ozonation	0.2	5 + 5
		0.45	10 + 10
		0.45/0.2	10 + 10
2.3	Final Reservoir	0.2	5 + 5
		0.45	10 + 10
		0.45/0.2	10 + 10

Treatment Steps

a) Ozonation b) Activated charcoal filtration c) Quartz sand filtration d) Chlorination

Retention time in DWDS until End Pipe about 1 day

Weather data obtained from: https://gate.meteoswiss.ch/idaweb;
obtained weather data period - 01.06.2020 - 15.07.2020

3. Luzern Spring Water

Sampling date	Nearest weather station information		
28.06.2020	Luzern	LUZ	665539 / 209847
	Precipitation during sampling		0.0 mm
	Precipitation 24h before sampling		0.0 mm

Number	Location	Filter (µm)	Filtered amount duplication 1 + duplication 2 (L)
3.1	Spring Water	0.2	2.5
		0.45	5
		0.45/0.2	6
3.2	Ozonation	0.2	5
		0.45	8
		0.45/0.2	10
3.3	Final Reservoir	0.2	5
		0.45	10
		0.45/0.2	10
3.4	End Pipe	0.2	5
		0.45	10
		0.45/0.2	10

Treatment Steps

a) Pre-filtration b) Ozonation c) Ceramic membrane filtration d) Activated charcoal filtration
e) UV-disinfection

Retention time in DWDS until End Pipe about 1 day

Weather data obtained from: https://gate.meteoswiss.ch/idaweb;
obtained weather data period - 01.06.2020 - 15.07.2020

4. Winterthur

Sampling date	Nearest weather station information		
07.07.2020	Winterthur	WIN	699845 / 259054
	Precipitation during sampling		0.0 mm
	Precipitation 24h before sampling		0.0 mm

Number	Location	Filter (µm)	Filtered amount duplication 1 + duplication 2 (L)
4.1	River Töss	0.2	1 + 1
		0.45	2 + 2
		0.45/0.2	4.5 + 4.5
4.2	Groundwater Protection Zone	0.2	4 + 4
		0.45	5 + 5
		0.45/0.2	5 + 5
4.3	Groundwater Reservoir	0.2	4 + 4
		0.45	5 + 5
		0.45/0.2	5 + 5
4.4	End Pipe	0.2	4 + 4
		0.45	5 + 5
		0.45/0.2	5 + 4.5

Treatment Steps
No treatment steps; using groundwater influenced by the river Töss

Retention time in DWDS until End Pipe	approximately 1.5 days

Weather data obtained from: https://gate.meteoswiss.ch/idaweb;
obtained weather data period - 01.06.2020 - 15.07.2020

5. Weinfelden

Sampling date	Nearest weather station information		
13.06.2020	Bischofszell	BIZ	737720 / 263610
	Precipitation during sampling		0.0 mm
	Precipitation 24h before sampling		0.0 mm

Number	Location	Filter (µm)	Filtered amount duplication 1 + duplication 2 (L)
5.1	River water (sidearm of the river Thur)	0.2	1
		0.45	1
		0.45/0.2	5
5.2	Groundwater	0.2	5
		0.45	10
		0.45/0.2	10
5.3	End Pipe	0.2	5
		0.45	7
		0.45/0.2	10

Treatment Steps
No treatment steps; using groundwater influenced by the river Thur

Retention time in DWDS until End Pipe	approximately 0.5 days

Weather data obtained from: https://gate.meteoswiss.ch/idaweb;
obtained weather data period - 01.06.2020 - 15.07.2020

6. Bischofszell

Sampling date	Nearest weather station information		
16.06.2020	Bischofszell	BIZ	737720 / 263610
	Precipitation during sampling		0.0 mm
	Precipitation 24h before sampling		0.0 mm

Number	Location	Filter (µm)	Filtered amount duplication 1 + duplication 2 (L)
6.1	River Thur	0.2	1.5 + 1.5
		0.45	2 + 2
		0.45/0.2	5 + 5
6.2	Groundwater	0.2	5 + 5
		0.45	10 + 10
		0.45/0.2	10 + 10
6.3	UV-treated	0.2	5 + 5
		0.45	10 + 10
		0.45/0.2	10 + 10
6.4	End Pipe	0.2	5 + 5
		0.45	10 + 10
		0.45/0.2	10 + 10

Treatment Steps

a) UV-disinfection; using groundwater influenced by the river Thur

Retention time in DWDS until End Pipe 2 - 3 days

Weather data obtained from: https://gate.meteoswiss.ch/idaweb;
obtained weather data period - 01.06.2020 - 15.07.2020

Annex 2. Protocol for DNeasy® PowerWater® Kit

- Solution PW1 must be warmed at 55 °C for 10 min.
- If Solution PW3 has precipitated, heat at 55 °C for 10 min to dissolve precipitate.

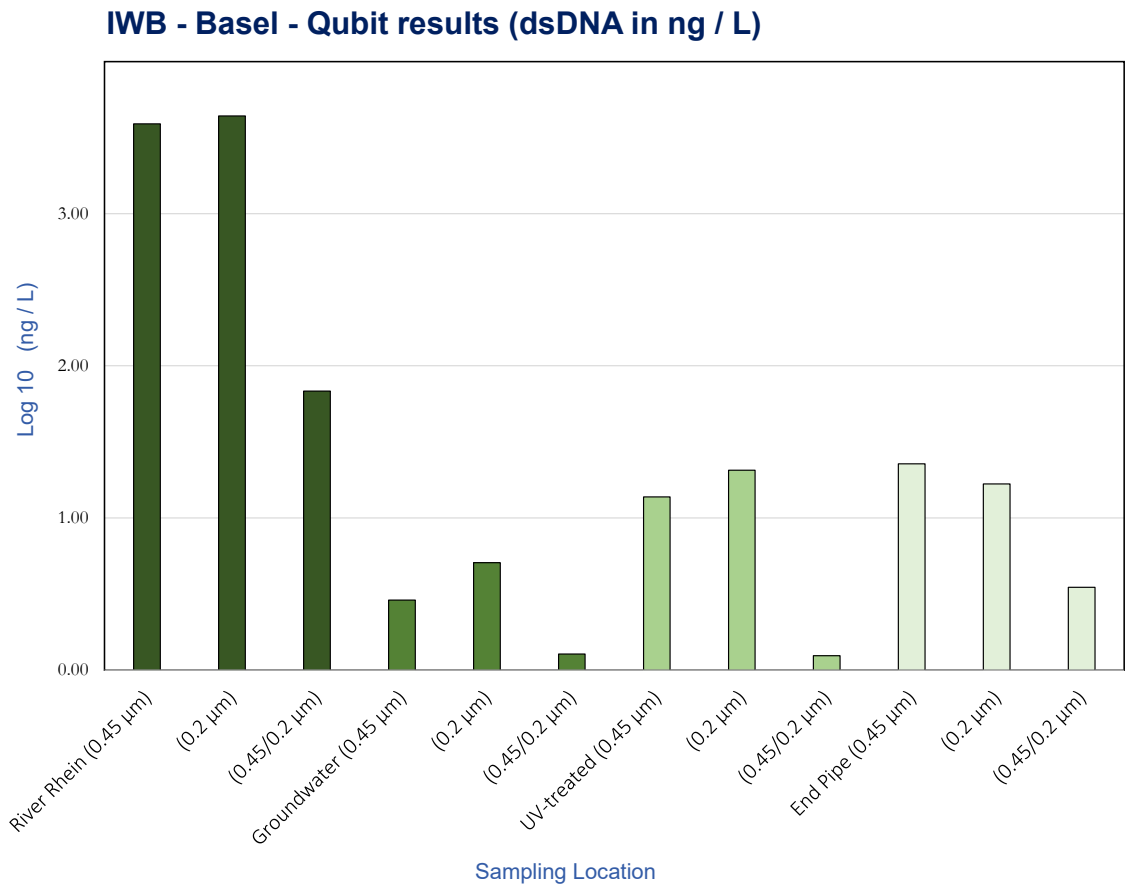
1. Using two sets of sterile forceps, pick up the white filter membrane at opposite edges and roll the filter into a cylinder with the top side facing inward. Insert the filter into a 15 ml PowerWater DNA Bead Tube.
2. Add 1 ml of Solution PW1 and provided beads to the Power Water DNA Bead Tube.
3. Beat the tubes with 6 m/s for 40 sec. Centrifuge the tubes with 4'000 rpm for 1 min at room temperature.
4. Transfer the supernatant to a provided clean 2 ml collection tube. Draw up the supernatant using a 1 ml pipette tip by placing it down into the beads. Recover as much as possible.
5. Centrifuge at 13'000 rpm for 1 min at room temperature.
6. Avoiding the pellet, transfer the supernatant to a provided clean 2 ml collection tube.
7. Add 200 µL of Solution IRS and vortex briefly to mix. Incubate at 4 °C for 5 min.
8. Centrifuge the tubes at 13'000 rpm for 1 min.
9. Avoiding the pellet, transfer the supernatant to a provided clean 2 ml collection tube.
10. Add 650 µL of Solution PW3 and vortex briefly to mix.
11. Load 650 µL of supernatant onto a MB Spin Column. Centrifuge at 13'000 rpm for 1 min. Discard the flow-through.
    Repeat this step until all the supernatant inside the 2 ml collection tube has been processed.
12. Place the MB Spin Column Filter into a provided clean 2 ml collection tube.
13. Add 650 µL of Solution PW4 (shake before use!). Centrifuge at 13'000 rpm for 1 min.
14. Discard the flow-through and add 650 µL of provided ethanol and centrifuge at 13'000 rpm for 1 min.
15. Discard the flow-through and centrifuge again at 13'000 rpm for 2 min.
16. Place the MB Spin Column into a provided clean 2 ml collection tube.
17. Add 50 µL of Solution EB to the center of the white filter membrane and incubate for 2 min at room temperature.
18. Centrifuge at 10'000 rpm for 2 min. Discard the MB Spin Column. The DNA is now ready for downstream applications.

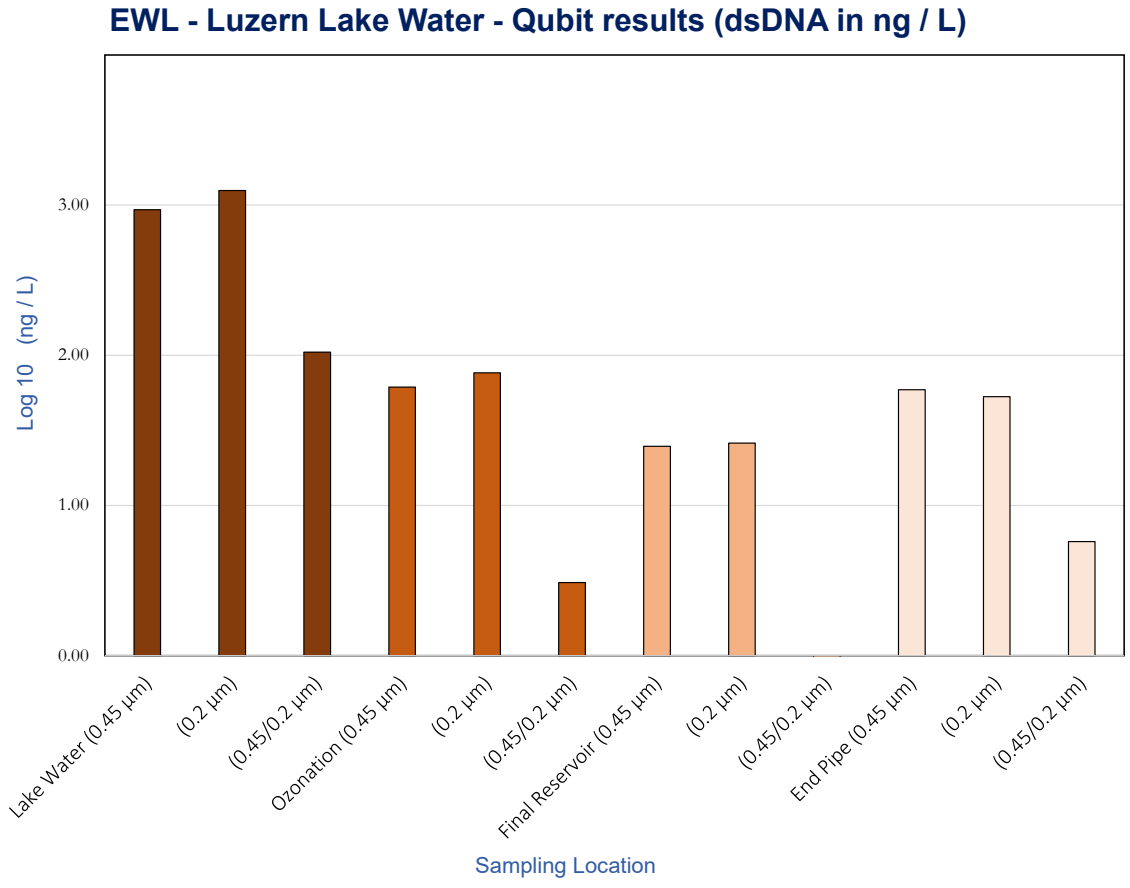
Annex 3. NanoDrop and Qubit results

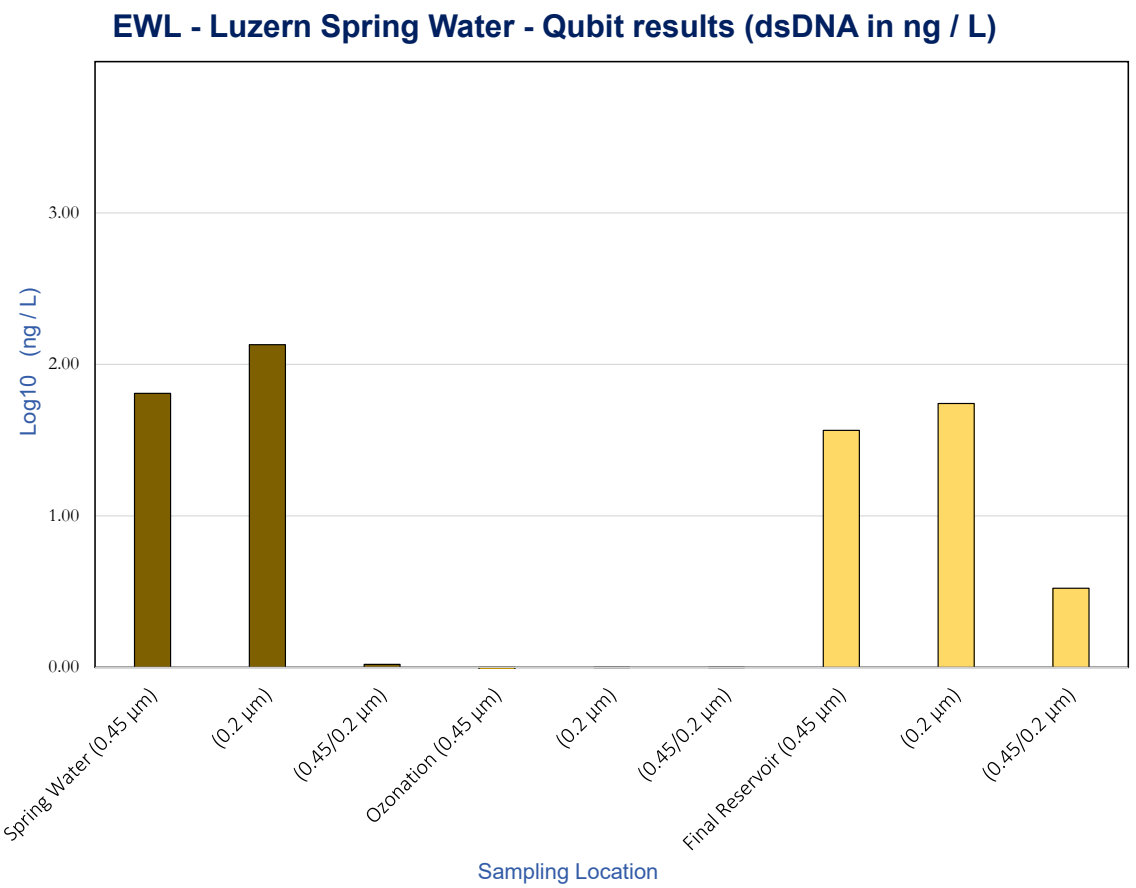
Sample name	dsDNA (ng / uL)	A260/A280	A260/A230
Basel_RiverRhein (0.45µm)	106.93	1.83	2.15
Basel_RiverRhein (0.2 µm)	143.06	1.85	1.88
Basel_RiverRhein (0.45/0.2 µm)	12.45	1.60	0.41
Basel_Gorundwater (0.45µm)	3.65	1.31	0.11
Basel_Gorundwater (0.2 µm)	3.50	1.16	0.40
Basel_Gorundwater (0.45/0.2 µm)	2.36	1.27	0.21
Basel_UV-disinfection (0.45µm)	6.07	1.65	0.06
Basel_UV-disinfection (0.2 µm)	6.16	1.58	0.24
Basel_UV-disinfection (0.45/0.2 µm)	3.56	1.37	0.18
Basel_EndPipe (0.45µm)	7.08	1.84	0.04
Basel_EndPipe (0.2 µm)	3.44	1.38	0.15
Basel_EndPipe (0.45/0.2 µm)	2.69	1.37	0.22
Luzern_Lake_RawWater (0.45µm)	68.10	1.83	1.93
Luzern_Lake_RawWater (0.2 µm)	46.63	1.83	1.56
Luzern_Lake_RawWater (0.45/0.2 µm)	12.16	1.74	0.58
Luzern_Lake_Ozonation (0.45µm)	16.36	1.73	0.75
Luzern_Lake_Ozonation (0.2 µm)	9.23	1.72	0.56
Luzern_Lake_Ozonation (0.45/0.2 µm)	2.80	1.73	0.02
Luzern_Lake_FinalReservoir (0.45µm)	6.95	1.65	0.25
Luzern_Lake_FinalReservoir (0.2 µm)	6.52	1.69	0.11
Luzern_Lake_FinalReservoir (0.45/0.2 µm)	3.07	1.15	0.33
Luzern_Lake_and_Spring_EndPipe (0.45µm)	15.82	1.78	0.78
Luzern_Lake_and_Spring_EndPipe (0.2 µm)	8.79	1.72	0.18
Luzern_Lake_and_Spring_EndPipe (0.45/0.2 µm)	3.31	1.47	0.07
Luzern_Spring_RawWater (0.45µm)	9.17	1.80	0.05
Luzern_Spring_RawWater (0.2 µm)	9.00	1.65	0.81
Luzern_Spring_RawWater (0.45/0.2 µm)	2.66	1.26	0.01
Luzern_Spring_Ozonation (0.45µm)	2.88	1.87	0.03
Luzern_Spring_Ozonation (0.2 µm)	2.40	1.27	0.03
Luzern_Spring_Ozonation (0.45/0.2 µm)	2.17	1.11	0.06
Luzern_Spring_FinalResevoir (0.45µm)	10.41	1.78	0.15
Luzern_Spring_FinalResevoir (0.2 µm)	8.58	1.68	0.14
Luzern_Spring_FinalResevoir (0.45/0.2 µm)	3.25	1.47	0.05
Winterthur_RiverTöss (0.45µm)	66.53	1.84	1.56
Winterthur_RiverTöss (0.2 µm)	49.07	1.83	0.36
Winterthur_RiverTöss (0.45/0.2 µm)	5.73	1.45	0.50
Winterthur_Groundwater_Protectionzone (0.45µm)	2.08	1.54	0.02
Winterthur_Groundwater_Protectionzone (0.2 µm)	3.58	1.43	0.61
Winterthur_Groundwater_Protectionzone (0.45/0.2 µm)	1.71	1.47	0.07
Winterthur_Groundwater_Reservoir (0.45µm)	3.28	1.56	0.22

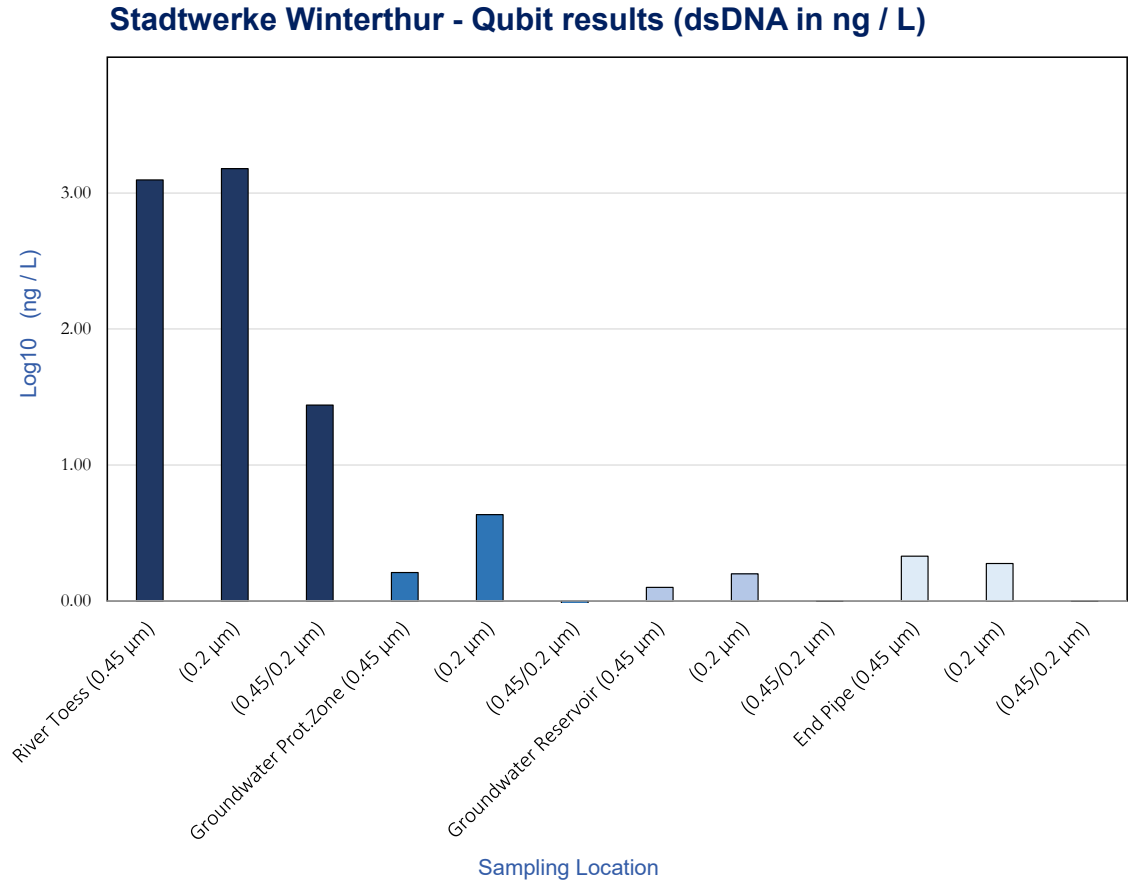
Winterthur_Groundwater_Reservoir (0.2 µm)	3.19	1.50	0.09
Winterthur_Groundwater_Reservoir (0.45/0.2 µm)	1.86	1.48	0.13
Winterthur_EndPipe (0.45µm)	3.20	1.47	0.05
Winterthur_EndPipe (0.2 µm)	2.34	1.17	0.22
Winterthur_EndPipe (0.45/0.2 µm)	1.47	1.06	0.05
Weinfelden_RiverWaterRaw (0.45µm)	99.99	1.85	2.15
Weinfelden_RiverWaterRaw (0.2 µm)	106.40	1.85	2.11
Weinfelden_RiverWaterRaw (0.45/0.2 µm)	8.30	2.04	1.31
Weinfelden_Groundwater (0.45µm)	2.02	2.28	0.34
Weinfelden_Groundwater (0.2 µm)	2.99	2.13	0.10
Weinfelden_Groundwater (0.45/0.2 µm)	1.58	2.70	0.38
Weinfelden_EndPipe (0.45µm)	34.89	1.92	1.87
Weinfelden_EndPipe (0.2 µm)	14.45	1.98	1.60
Weinfelden_EndPipe (0.45/0.2 µm)	2.79	2.55	0.05
Bischofszell_RiverThur (0.45µm)	96.93	1.85	1.74
Bischofszell_RiverThur (0.2 µm)	54.33	1.85	2.04
Bischofszell_RiverThur (0.45/0.2 µm)	10.35	1.76	1.21
Bischofszell_Groundwater (0.45µm)	4.19	1.79	0.10
Bischofszell_Groundwater (0.2 µm)	3.09	1.83	0.04
Bischofszell_Groundwater (0.45/0.2 µm)	2.16	1.82	0.10
Bischofszell_UV-disinfection (0.45µm)	4.06	1.56	0.13
Bischofszell_UV-disinfection (0.2 µm)	3.90	1.38	0.21
Bischofszell_UV-disinfection (0.45/0.2 µm)	4.20	1.35	0.26
Bischofszell_EndPipe (0.45µm)	14.24	1.82	0.26
Bischofszell_EndPipe (0.2 µm)	3.17	1.58	0.16
Bischofszell_EndPipe (0.45/0.2 µm)	7.53	1.66	0.50
Dup_Basel_RiverRhein (0.45µm)	128.68	1.87	2.21
Dup_Basel_RiverRhein (0.2 µm)	122.47	1.88	1.66
Dup_Basel_RiverRhein (0.45/0.2 µm)	5.51	2.20	1.48
Dup_Basel_Gorundwater (0.45µm)	3.03	1.86	0.50
Dup_Basel_Gorundwater (0.2 µm)	3.22	1.73	0.43
Dup_Basel_Gorundwater (0.45/0.2 µm)	2.09	2.32	0.46
Dup_Basel_UV-disinfection (0.45µm)	4.93	2.26	1.22
Dup_Basel_UV-disinfection (0.2 µm)	2.43	2.03	0.78
Dup_Basel_UV-disinfection (0.45/0.2 µm)	1.68	2.44	0.47
Dup_Basel_EndPipe (0.45µm)	7.54	1.97	1.06
Dup_Basel_EndPipe (0.2 µm)	3.10	1.88	0.79
Dup_Basel_EndPipe (0.45/0.2 µm)	3.00	1.99	0.59
Dup_Luzern_Lake_RawWater (0.45µm)	83.18	1.89	2.00
Dup_Luzern_Lake_RawWater (0.2 µm)	53.37	1.89	1.95
Dup_Luzern_Lake_RawWater (0.45/0.2 µm)	11.42	1.99	1.14
Dup_Luzern_Lake_Ozonation (0.45µm)	12.62	1.95	0.48
Dup_Luzern_Lake_Ozonation (0.2 µm)	10.13	2.03	0.38
Dup_Luzern_Lake_Ozonation (0.45/0.2 µm)	3.68	2.14	0.19

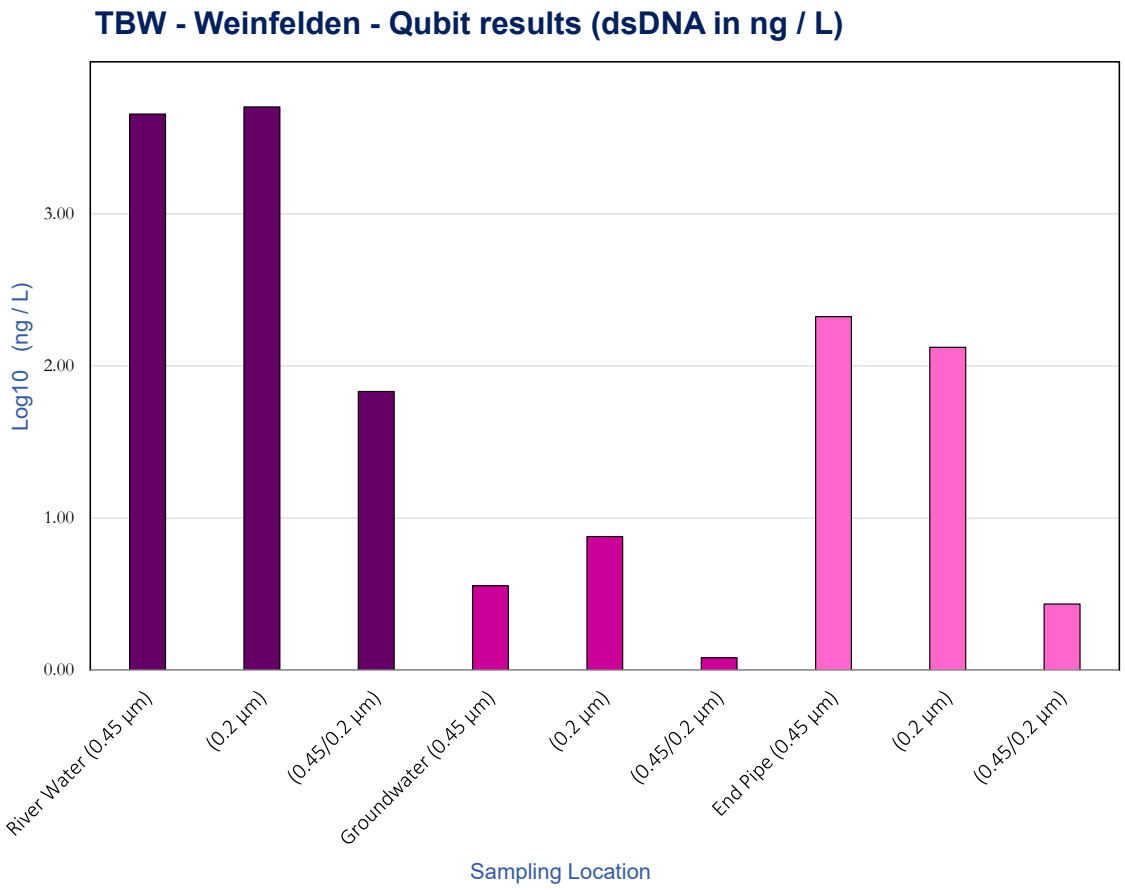
Dup_Luzern_Lake_FinalReservoir (0.45µm)	7.17	2.04	0.33
Dup_Luzern_Lake_FinalReservoir (0.2 µm)	3.64	3.00	0.08
Dup_Luzern_Lake_FinalReservoir (0.45/0.2 µm)	2.74	2.38	0.48
Dup_Winterthur_RiverTöss (0.45µm)	42.95	1.87	1.83
Dup_Winterthur_RiverTöss (0.2 µm)	18.59	1.85	1.63
Dup_Winterthur_RiverTöss (0.45/0.2 µm)	4.27	2.10	1.15
Dup_Winterthur_Groundwater_Protectionzone (0.45µm)	3.69	1.94	0.59
Dup_Winterthur_Groundwater_Protectionzone (0.2 µm)	2.38	3.00	0.28
Dup_Winterthur_Groundwater_Protectionzone (0.45/0.2 µm)	2.88	1.74	0.58
Dup_Winterthur_Groundwater_Reservoir (0.45µm)	1.76	3.15	0.59
Dup_Winterthur_Groundwater_Reservoir (0.2 µm)	3.20	1.86	0.59
Dup_Winterthur_Groundwater_Reservoir (0.45/0.2 µm)	2.30	2.00	0.46
Dup_Winterthur_EndPipe (0.45µm)	2.61	2.02	0.47
Dup_Winterthur_EndPipe (0.2 µm)	2.87	1.94	0.24
Dup_Winterthur_EndPipe (0.45/0.2 µm)	1.81	2.41	0.48
Dup_Bischofszell_RiverThur (0.45µm)	94.40	1.92	1.83
Dup_Bischofszell_RiverThur (0.2 µm)	56.88	1.96	1.47
Dup_Bischofszell_RiverThur (0.45/0.2 µm)	11.13	2.34	0.04
Dup_Bischofszell_Groundwater (0.45µm)	4.47	1.84	0.66
Dup_Bischofszell_Groundwater (0.2 µm)	4.44	1.92	0.39
Dup_Bischofszell_Groundwater (0.45/0.2 µm)	3.72	1.80	0.12
Dup_Bischofszell_UV-disinfection (0.45µm)	5.16	1.65	0.10
Dup_Bischofszell_UV-disinfection (0.2 µm)	2.95	1.85	0.02
Dup_Bischofszell_UV-disinfection (0.45/0.2 µm)	2.12	2.21	0.30
Dup_Bischofszell_EndPipe (0.45µm)	14.54	1.92	0.29
Dup_Bischofszell_EndPipe (0.2 µm)	7.78	1.89	0.68
Dup_Bischofszell_EndPipe (0.45/0.2 µm)	3.63	1.79	0.19
BLANK	-0.22	0.44	0.38

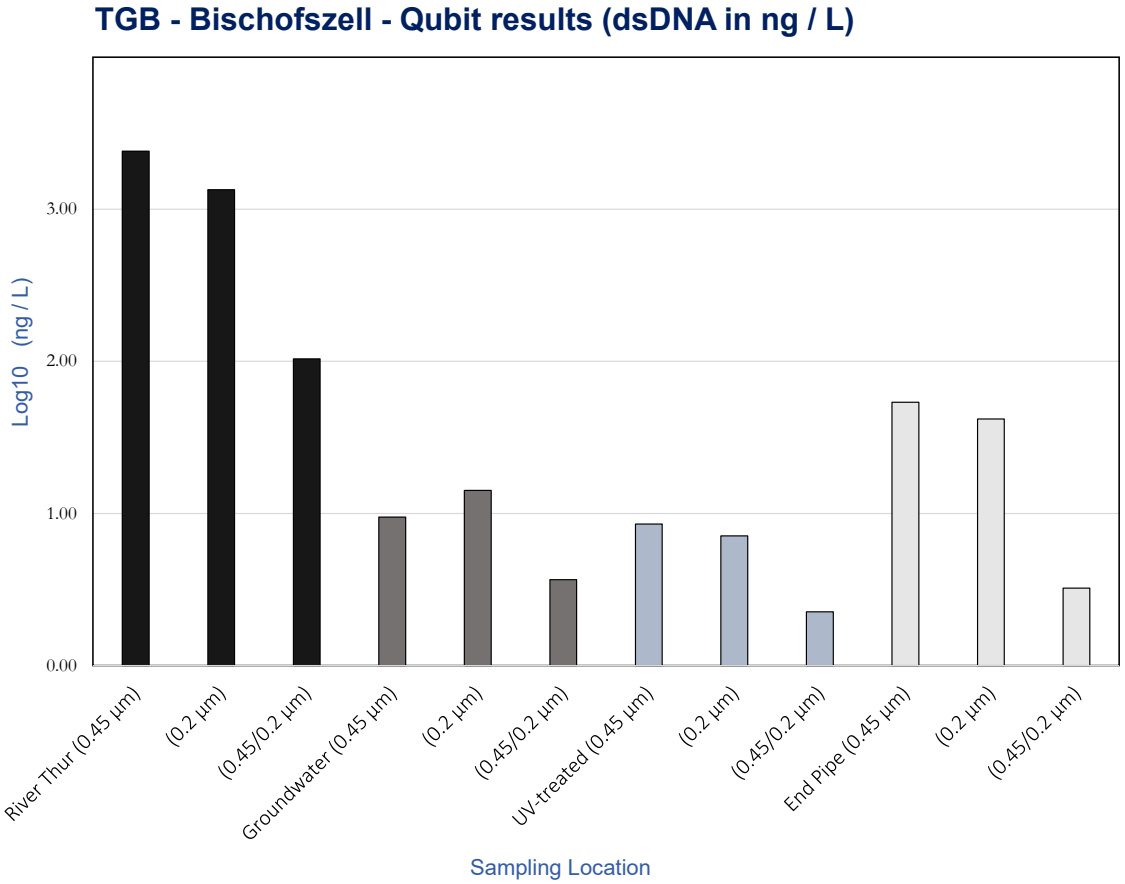

IWB - Basel - Qubit results (dsDNA in ng / L)
Log 10 (ng / L)
3.00
2.00
1.00
0.00
River Rhein (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Groundwater (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
UV-treated (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
End Pipe (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Sampling Location


EWL - Luzern Lake Water - Qubit results (dsDNA in ng / L)
Log 10 (ng / L)
3.00
2.00
1.00
0.00
Lake Water (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Ozonation (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Final Reservoir (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
End Pipe (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Sampling Location


EWL - Luzern Spring Water - Qubit results (dsDNA in ng / L)
Log10 (ng / L)
3.00
2.00
1.00
0.00
Spring Water (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Ozonation (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Final Reservoir (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Sampling Location


Stadtwerke Winterthur - Qubit results (dsDNA in ng / L)
Log10 (ng / L)
3.00
2.00
1.00
0.00
River Toess (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Groundwater Prot.Zone (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Groundwater Reservoir (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
End Pipe (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Sampling Location


TBW - Weinfelden - Qubit results (dsDNA in ng / L)
Log10 (ng / L)
3.00
2.00
1.00
0.00
River Water (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Groundwater (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
End Pipe (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Sampling Location


TGB - Bischofszell - Qubit results (dsDNA in ng / L)
Log10 (ng / L)
3.00
2.00
1.00
0.00
River Thur (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Groundwater (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
UV-treated (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
End Pipe (0.45 µm)
(0.2 µm)
(0.45/0.2 µm)
Sampling Location