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APPLICATION OF ONLINE-FLOWCYTOMETRY IN DRINKING WATER TREATMENT

Master thesis

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submitted by:

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Abbreviations

DMSO	Dimethyl Sulfoxide
FL1	Fluorescence signal 1 (green fluorescence)
FL2	Fluorescence signal 2 (orange fluorescence)
FL3	Fluorescence signal 3 (red fluorescence)
FSC	Forward scatter
HNA	High nucleic acid cells
HPC	Heterotrophic plate counts
ICC	Intact Cell Count
ID	Inside diameter
LNA	Low nucleic acid cells
OBA	Online Bacteria Analyzer
OD	Outside diameter
PP	Polypropylene
PTFE	Polytetrafluoroethylene
SG	SYBR Green
SGPI	SYBR Green propidium iodide
SSC	Side scatter
TCC	Total Cell Count
TOC	Total organic carbon
TON	Total organic nitrogen
UV-C	Short-wavelength ultraviolet

Abstract

The primary target of this thesis was finding the confines of the application of Online Bacteria Analyzer (OBA) designed and provided by METANOR AG (Switzerland).

In order to understand the main processes taking place and exploring all the potential uses of the equipment, laboratory and field tests were conducted from April until December 2017.

Within laboratory research, the following parameters were chosen to be analysed: the incubation time of staining and the effect of system cleaning on the obtained results. The results showed that the incubation time for TCC determination with stain SG should be adjusted at 5 min and 10 min of incubation time for ICC with SGPI. Also, the effect of cleaning the system with pure water or with cleaning solution on the results was confirmed. The number of detected bacterial cells after each cleaning of the system was reduced and therefore the first three measurements after cleaning did not represent the real amount of bacteria in the water.

Stand trial experiments for analysing growth potential of bacteria in UV-C processed water and the real-time monitoring of raw water were performed. The results from the stand trial experiments showed that pure water cleaning application every 24 h is necessary to avoid the growth of bacterial cells in the system to get stable results. In the real-time experiments, it is required to perform cleaning with cleaning solution every 24 h if the water quality is stable and every 16 h if the water quality is expected to change (e.g. when another water source is used). The results obtained in the real-time experiments showed that the online-flow cytometer could be applied in the field research (at the waterwork). For obtaining stable results, it is necessary to perform the system cleaning with pure water or cleaning solution and to follow the incubation time of staining.

Kurzfassung

Das Hauptziel dieser Arbeit war es, die Grenzen der Anwendung des Online-Durchflusszytometers Online Bacteria Analyzer, das von METANOR AG (Schweiz) entwickelt und zur Verfügung gestellt wurde aufzuzeigen.

Um die wichtigsten Prozesse zu verstehen und alle möglichen Verwendungen der Geräte zu erforschen, wurden Labor- und Feldtests von April bis Dezember 2017 durchgeführt.

Im Rahmen der Laborforschung wurden die folgenden Parameter zur Analyse ausgewählt: die Inkubationszeit der Färbung und die Auswirkung der Systemreinigung auf die Ergebnisse. Die Ergebnisse zeigten, dass die Inkubationszeit für die TCC-Bestimmung mit Farbstoff SG für 5 min und 10 min für ICC-Bestimmung mit Farbstoff SGPI eingestellt werden sollte. Auch wurde die Wirkung der Reinigung des Systems mit reinem Wasser oder mit Reinigungslösung auf die Ergebnisse bestätigt. Die Anzahl der Bakterienzellen nach jeder Reinigung des Systems wurde verringert, und daher repräsentieren die ersten drei Messungen nicht die tatsächliche Menge der im Wasser vorhandenen Bakterien.

Zur Analyse des Wachstumspotentials von Bakterien wurden Standversuchsexperimente in UV-C-behandeltem Wasser und Durchflussanalysen von Rohwasser durchgeführt. Die Ergebnisse der Standversuchsexperimente zeigten, dass eine Reinigung mit reinem Wasser alle 24 h erforderlich ist, um das Wachstum von Bakterienzellen im System zu vermeiden und um stabile Ergebnisse zu erhalten. In den Durchflussanalysen ist es erforderlich, die Reinigung mit Reinigungslösung alle 24 h durchzuführen, wenn die Wasserqualität stabil ist, und alle 16 h, wenn sich die Wasserqualität voraussichtlich ändern wird (z.B. Verwendung einer anderen Wasserquelle). Die Anwendung von OBA zeigte, dass die Online-Durchflusszytometrie (OBA) in der Wasserversorgung eingesetzt werden kann. Um stabile Ergebnisse zu erhalten, ist es notwendig, die Systemreinigung mit reinem Wasser oder mit Reinigungslösung durchzuführen und die Inkubationszeit für die Färbung anzupassen.

1 Introduction

The monitoring of water quality should be continuously checked because of numerous infections that could occur through bacterial pollution (José Figueras and Borrego, 2010).

Flow cytometry is a modern tool equipped with an optical system that detects several parameters of cells (size, fluorescence). Flow cytometry is used for cells detection and quantification (Boujard et al., 2014).

The benefits of applying the flow cytometer are: rapid characterisation and counting of cells in a short time and opportunity to obtain various cell parameters. The results could be displayed in several ways, depending on the existent software in the flow cytometer (Luttmann et al., 2014). Measurements done with the help of flow cytometer are advantageous because information about the size and amount of cells is provided (Broger et al., 2011).

Studies used various water samples: native bacterial growth in water, pollution of drinking water with wastewater, household drinking water and the mix of two different drinking water types. This method can be used for drinking water assessment in distribution networks due to stable results (Prest et al., 2013).

On the market there are some companies that construct flow cytometers. The working principle of all flow cytometers is the same; it is sometimes the equipment and possibilities of measurements that can differ (Luttmann et al., 2014).

This research was provided with an entirely new online-flow cytometer named Online Bacteria Analyzer (OBA) designed by METANOR AG (Switzerland). This equipment stands out from the flow cytometers that currently existing on the market. Online Bacteria Analyzer has installed automated sample ports for taking measurements in the flow. Incubation provided internally and time of incubation can be changed depending on the applied settings. Within 15 minutes the result of the water sample is received. Quantification of the obtained events is done with the internal software designed by METANOR AG (Switzerland). Equipment has the internet connection that allows to save data on the external server and obtain notification e-mails when any problems occur.

2 Objectives

This thesis aims to check the application of the online-flow cytometry in the continuous flow and the stability of obtained measurements by applying new equipment. The monitoring of drinking water in real-time was done with an automated sampling system. Different water samples were analysed using the new equipment named Online Bacteria Analyzer (OBA), created by METANOR AG. Therefore, specific conditions, settings, usability and effectiveness of the flow cytometer were established in the water analysis.

Changes in microbiological parameters such as TCC and ICC were monitored, stored in the internal and external software and analysed. Water samples were examined for bacterial growth by a stand trial experiment and direct measurements of raw and drinking water by the real-time test were performed. Both investigations were firstly done in the lab scale and afterwards applied in the field (at the waterwork).

Areas of research considering such observations:

1. In the lab scale research:
   - Investigation of construction features of equipment and definition of processes that take place.
   - Clarification of specific preparation procedures for required liquids and stains and assessment of average consumption of fluids.
   - Defining sample incubation time, cleaning of the system (equipment), specifying measuring settings for uninterrupted analysis of samples and calibration of the device.
   - Setting the measuring plot area (Gate) for OBA. The created gate provides information about the total cell count (TCC), low nucleic acid cells (LNA) and high nucleic acid cells (HNA). The plot area was chosen to be comparable to another applied flow cytometer.
2. In the field research:
   - The stand trial experiment explores growth potential of cells in drinking water after UV-C disinfection.
   - The real-time experiment for monitoring microbiological changes in raw water with continuous sampling at fixed time intervals (15 minutes – 1 hour).
   - Description of problems in the software configurations and indicating possible improvements.

All measurements in the lab scale and at the waterwork were collected and analysed from April 2017 until December 2017. In this thesis, the methods of analysis were defined, and the possibility of utilizing the new equipment in the field was checked.

The results of the work are depicted in the consecutive order. The final part of this master thesis provides conclusions and future research recommendations using Online Bacteria Analyzer.

3 Fundamentals

3.1 Conventional and modern methods of cells detection

There is a considerable amount of microbiological samples investigated using conventional culture-based methods. Flow cytometry allows doing microbial analysis in real-time, but because of high cost and difficulty of instrumentation, it was not used in the routine microbial analysis (Veal et al., 2000).

Flow cytometry is an alternative device to heterotrophic plate counts (HPC) that offers to monitor drinking water quality (Hoefel et al., 2003). In drinking water, not all cells can be identified by traditional cultivation HPC method (Hammes et al., 2008). Flow cytometer detected cells even if there was no growth of bacteria by plate count technique (Pianetti et al., 2005).

The flow cytometry provides quantification and characterisation of cells by various parameters, such as enzyme activity, membrane potential and integrity (Pianetti et al., 2005). The standard bacteria analysis with HPC method takes longer (1 day), which could be critical for water suppliers (Van Nevel et al., 2017) whereas with the flow cytometer, it takes only 20 minutes (Hammes et al., 2008). Moreover, flow cytometry is self-sufficient and needs no cultivation, which makes this technique more attractive in comparison to traditional plate methods (Pianetti et al., 2005). The benefit of FCM is in measurement error of less than 5% in comparison to traditional HPC method that has a measurement error of more than 30% (Hammes et al., 2008).

Van Nevel et al. (2016) concluded that drinking water analysis in the network was possible only with FCM because the cultivation method did not produce any relevant data about bacterial growth.

3.2 Flow cytometry

The first commercially available flow cytometers without fluorescence were used in the 1970's in clinical laboratories for determining the number of blood cells. Their simple usage and accuracy of results led to their widespread use. Modern equipment called flow cytofluorometers utilises fluorescence. Almost every scientific research in cell biology applies flow cytometers in their studies, due to the accuracy of results (Nunez, 2001).

3.2.1 Principles of flow cytometry measurement

The flow cytometry is based on the laser that is used to estimate cells in liquids. The use of flow cytometer in medical research owes to the rapid estimation of thousands of cells in several seconds (Jaroszeski and Radcliff, 1999).

Measuring with the flow cytometer requires the next steps:

1. Sample preparation;
2. Set the settings in the flow cytometer;
3. Sample measuring;
4. Evaluation of obtained signals (Luttmann et al., 2014).

The working principle of flow cytometers is: the cell suspension is pumped under pressure into the analysis area. The suspension forms one continuous flow and is directed to the area where the laser is situated. Cells are going in the row and pass the laser, where fluorescence of cells is estimated. Obtained signals are transferred to computer software where subsequent calculations of cells is done. The presentation of received data is in the form of diagrams and histograms (Boujard et al., 2014).

All flow cytometers have the same principal elements that are shown in the Figure 1:

- Fluidic system – responsible for sample transfer to analysis area.
- Illumination system – from the fluidic system the sample is transferred to the region equipped with the laser.
- Optical and electronics system – the number of obtained fluorescence and the light would be accumulated and converted into electronic signals.
- Data storage and computer control system – electronic signals are transformed into data and stored. With the help of software calculations and histograms are produced (Jaroszeski and Radcliff, 1999).

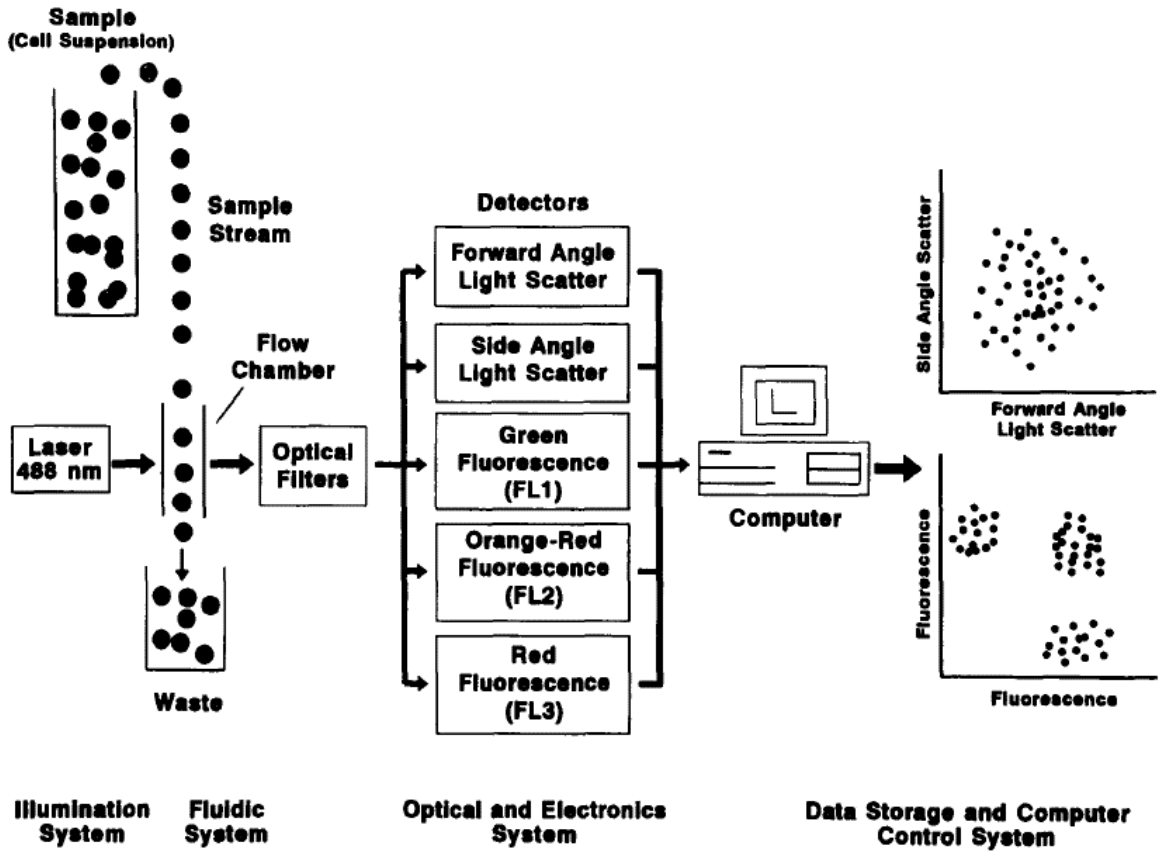

Figure 1: The principal elements of the flow cytometer (Jaroszeski and Radcliff, 1999)

The online flow cytometer consists of elements that are shown in Figure 2:

- (A) Sampling and staining injection system with next incubation.
- (B) Flow cytometer section.
- (C) Control module and data handling system (Hammes et al., 2012).

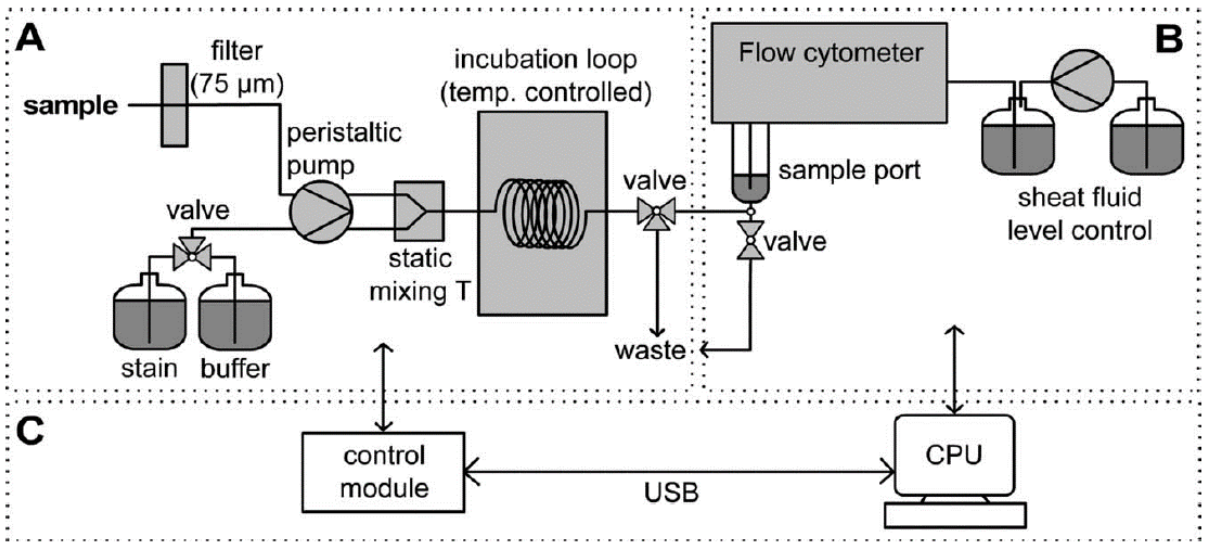

Figure 2: Overview of the main elements of the online flow cytometer (Hammes et al., 2012)

The flow cytometer employs plural modes for each cell measurement:

- Forward scatter intensity (FSC) is nearly comparable to cell size.
- Side scatter intensity (SSC) is relatively equal to the number of granular formations inside the cell.
- Fluorescence intensities estimated at different wavelengths (Nunez, 2001).

The most frequently used fluorescence detectors in FCM of water are:

- Green fluorescence FL1 channel (525 nm).
- Orange fluorescence FL2 channel (585 nm)
- Red fluorescence FL3 channel (>670 nm) (Pianetti et al., 2005).

Figure 3 details how stained cells in the laminar flow pass the laser area. For each different fluorescence dye specific signal is obtained. Additionally, information about cell size and granularity is received. (Luttmann et al., 2014).

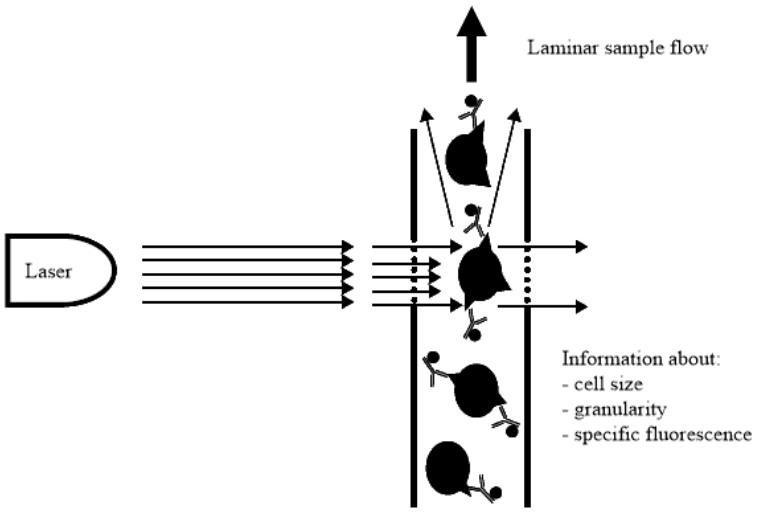

Figure 3: The principle of a flow cytometric measurement (Luttmann et al., 2014)

The obtained data is interpreted using internal flow cytometers software. Transfer of collected data and following analysis can be done on a separate computer. This step is necessary when the amount of collected data is tremendous (Bakalar and Tomas, 2016).

3.2.2 Problems of applying flow cytometry in the researches

The different scientific research was performed by applying flow cytometry in water analysis. For estimating bacterial cells, various procedures in researches were conducted. The literature analysis of the researches revealed the major problems in:

1. Gating procedure:
   - Dividing of TCC into two clusters to HNA and LNA cells (Liu et al., 2013);
   - Determination and selection of LNA and HNA clusters (Prest et al., 2013);
   - The way of data presentation (Luttmann et al., 2014).
2. Staining and incubation procedures:
   - Determination of incubation time (Prest et al., 2013);
   - Determination of incubation temperature (Hammes et al., 2012);
   - Protection samples from the light and ensuring a stable incubation temperature (Gillespie et al., 2014);

3.2.3 Gating procedure (TCC, LNA, HNA)

Water samples analysis with FCM by using fluorescent stains provides information about total cell count (TTC), bacterial properties and viability (Veal et al., 2000). FCM measurements are usually used for the characterisation of high and low nucleic acid (Wang et al., 2009).

The gating procedure for TCC, LNA and HNA is depicted in Figure 4.

Figure 4 shows that:

- (A) The fixed gate (red dotted line) is applied for the counting of bacterial cells (TCC) and exclusion of background in drinking water.
- (B) Determination and selection of LNA and HNA clusters (Prest et al., 2013).

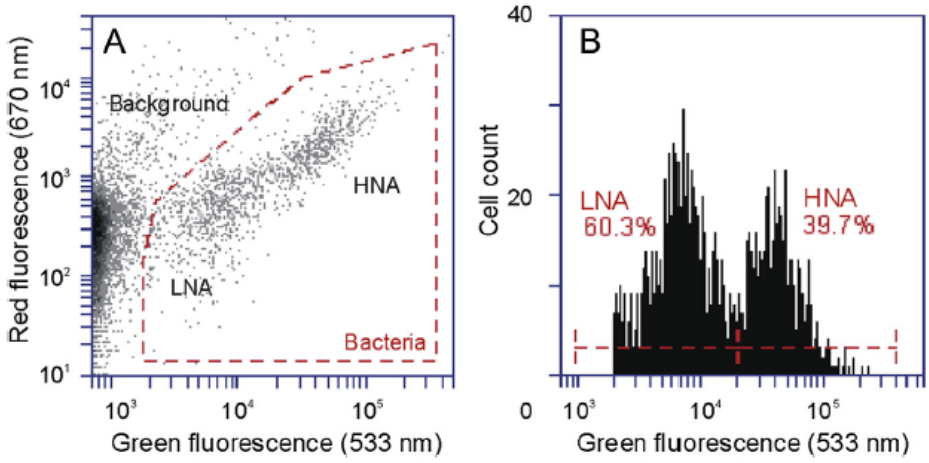

Figure 4: Data selection and gating procedure (Prest et al., 2013).

The data could be presented in different types of plots:

1. Histogram plot. The simplest way of data presentation is the histogram in which the fluorescence signals are distributed depending on the frequency of the obtained signal. Depending on the intensity of signals results can be presented in a logarithmic or linear way. The advantage of logarithmic data presentation lies in the high-grade resolution of the obtained signal (Luttmann et al., 2014).
2. Dot plot (example is shown in Figure 5). The cell signals that overlap in the histogram can be presented in the two-dimensional dot plot, for a better characterisation of microorganisms. (Luttmann et al., 2014).

3. Density plot. Overlaid points in dot plot diagram can be coloured for making the presentation of data more visible based on the intensity of the received signal. The image in the density plot is black and white with the possibility of shading (Luttmann et al., 2014).

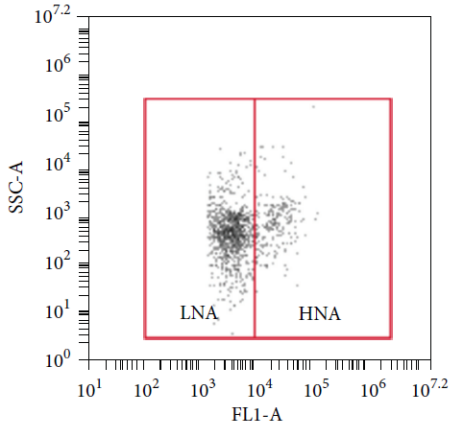

Figure 5: Dot plot with TTC, LNA and HNA counts of cells (Liu et al., 2013)

The flow cytometric measurements in Figure 5 show a consecutive dividing of total bacteria cells into two clusters with HNA and LNA cells (Liu et al., 2013).

FCM was used in water analysis for the detection of areas with bacterial and viral cells. Measurements showed a good separation of bacteria and viruses. (Huang et al., 2016).

Figure 6 shows:

- Plot with the single parameter was used for distinguishing peaks of cells (M1 and M2 regions). Density plot was used for identifying various cell types.
- Different gates were used for quantifying the number of counts in separate groups of cells. Viral cells were determined in R1 area, and bacterial cells in R2 area (Huang et al., 2016).

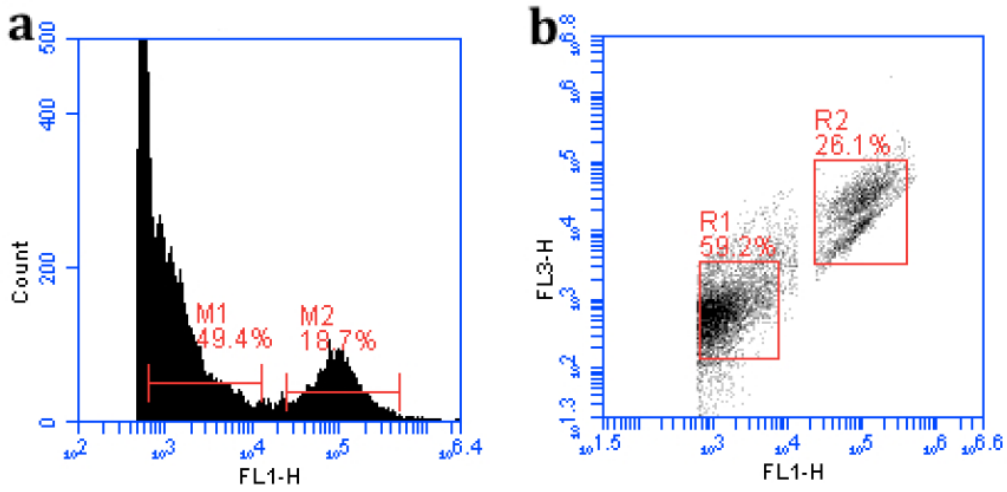

Figure 6: Interpretation of FCM measurements with application of a the single parameter (a) and density plot (b) (Huang et al., 2016)

FCM software used for processing all data from the measured samples. Calculation of the number of LNA and HNA cells could be done after placing the gate under green fluorescence. Comparison of obtained data based on the total cell count (TCC) and the number of HNA cells in the water samples (Prest et al., 2013).

3.2.4 Staining and incubation procedures

For measuring water samples and obtaining relevant data parameters such as staining temperature and staining time are fundamental (Prest et al., 2013). SYBR Green I and propidium iodide stains contributed to the fast detection and counting of a large number of cells in water samples (Pianetti et al., 2005).

The staining and incubation procedures in scientific research varied and have been done under various conditions. Examples include:

- Van Nevel et al. (2016) used an incubation time of 13 minutes at 37 °C for total cell count with SYBR Green I and for intact cell count with SYBR Green I propidium iodide;
- Prest et al. (2016) warmed samples for 5 minutes at 35 °C. Afterwards, samples (500 µL) were stained and incubated for 10 minutes at 35 °C;
- Hammes et al. (2012) conducted research with staining at a temperature ranging between 5 and 50 °C and with an incubation time of 5 minutes (effects of incubation are shown in Figure 7). Another experiment from the same source was done using staining: One sample was stained at room temperature for 15 minutes, and another one at 40 °C for 5 minutes;
- Prest et al. (2013) employed staining at a temperature of 4, 22 and 35 °C respectively. Staining time varied between 5, 10 and 15 minutes (Consequences of staining methods are shown in Figure 8);
- Gillespie et al. (2014) set the incubation time at 15 minutes and the incubation temperature at 30 °C. To protect samples from the light and ensure a stable incubation temperature aluminium foil was used for covering the samples;
- A different research was done by Park et al., (2016) at a waterwork in South Korea by applying the staining procedure with SYBR Green I and propidium iodide. After staining, samples were incubated for 15 minutes at 36 °C.

Data obtained from the measurements of a similar sample under varying staining and incubation temperatures showcases the following:

1. Hammes et al. (2012) concluded that the number of detected cells at incubation temperatures below 30 °C varied considerably (shown in Figure 7). Figure 7 shows that the measured total cell count and green fluorescence depend on incubation temperature of the stained sample. After data analysis, Hammes et al. (2012) fixed the incubation temperature of staining at 40 °C for 5 minutes.
   Figure 7 shows: (○) TTC, total cell count; (Δ) FL1, green fluorescence; (□) FSC, forward scatter (Hammes et al., 2012).

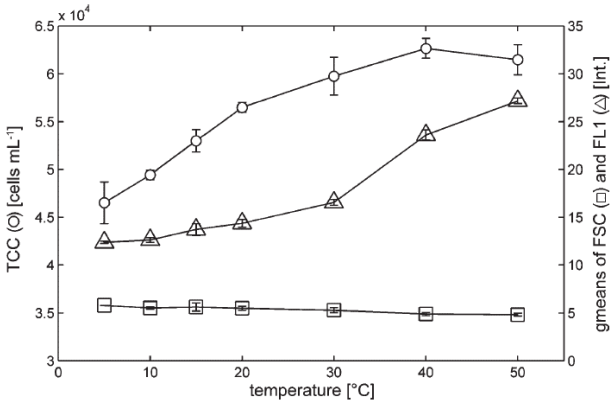

Figure 7: Effects of 5 minutes incubation at various staining temperatures (Hammes et al., 2012)

2. Prest et al. (2013) showed that staining temperature and staining time directly influenced results (shown in Figure 8). Cell concentrations varied depending on staining temperature. When the staining temperature was at 4 °C, the cell concentrations were considerably lower in comparison to those at 35 °C. Cell concentrations of measured samples were progressing with the increase of staining time (Prest et al., 2013).
   According to Prest et al.'s (2013) research the staining protocol has the following specifications: 5 minutes pre-incubation at 35 °C, staining and additional 10 minutes incubation at 35 °C.
   Figure 8 shows:
   - (A) Data comparison of green fluorescence histograms by various temperatures (4, 22, 35 °C) and at 10 minutes fixed staining time.
   - (B) Influence of staining temperature on cell concentrations of TTC, LNA and HNA cells.
   - (C) Mixed-effects of TTC and percentage of HNA. The markers display the incubation time (min), and the error bars represent the standard deviation on triplicate measurements (Prest et al., 2013).

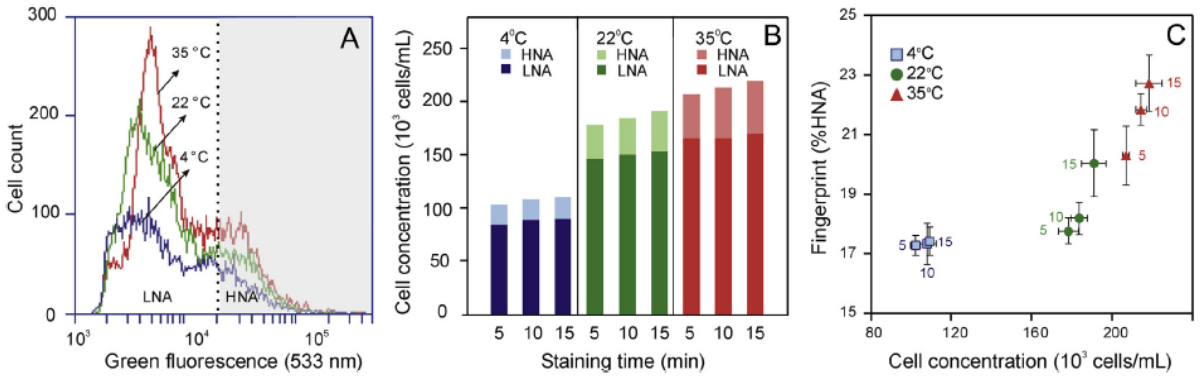

Figure 8: Consequences of staining methods on the same water sample (Prest et al., 2013)

3.3 Bacterial growth potential in water

Bacterial growth potential in water is measured for the purposes of:

- estimating the degree of disinfection (Ramseier et al., 2011);
- measuring the destruction of the membrane (Ramseier et al., 2011);
- determination and differentiation of diverse water samples (tap, bottled, river, pond and wastewaters) (Prest et al., 2013);
- measuring the regrowth of bacteria in drinking water samples (Gillespie et al., 2014).

For estimating bacterial growth potential different research has been done:

- by drinking water samples treated with ozone, chlorine and chlorine dioxide (Ramseier et al., 2011);
- by comparing cell concentrations of tap and bottled water (Prest et al., 2013);
- through a one-year water research at two waterworks in the Netherlands at different temperatures (Liu et al., 2013);
- by drinking water samples, coming from Scottish reservoirs and treated with a disinfectant solution (chlorine or chloramine). One drinking water sample was treated with chlorine, and another two with chloramine (Gillespie et al., 2014).

For performing measurements, different researches used different procedures:

- Collected water samples were transported in 500 ml bottles, incubated at 22 °C and measured daily for ten days. Results are shown in Figure 9 (Gillespie et al., 2014);
- Measurements of membrane destruction were performed by using FCM with two staining procedures. SYBR Green I has been used for determining total cell count (TCC) and SYBR Green I and propidium iodide for estimating the penetration of water through the membrane (Ramseier et al., 2011).

Research outcomes showed the following:

- small concentrations of chlorine could cause in order to the cell membrane. A reduction of the total cell count (TCC) has been observed in the case of water treated with ozone. In water treated with chlorine dioxide cells in the HNA region were considerably faster-damaged than those in the LNA section. Water with chlorine had only a tiny impact on membrane destruction in the HNA and LNA fields (Ramseier et al., 2011);
- bottled water had a higher percentage of HNA cells in comparison to tap water. The cell concentrations in tap and bottled waters were lower in contrast to river, pond and wastewaters (Prest et al., 2013).
- at temperatures above 15 °C there were considerably more bacterial cells than at temperatures below 15 °C (Liu et al., 2013);
- insignificant variation in TCC (shown in Figure 9). One sample received from waterwork (from System II) showed no changes in TCC for ten days. Measurement of ICC showed slight growth potential for all three samples. The analysis of samples received from system I and system II reported a slight fold increase in ICC on day 7 and day ten. Collected sample from system III showed a more considerable fold increase in ICC. Changes of TOC, TON, NH_4^+ (ammonium) and PO_4^{3-} (phosphate) concentrations on days 0, 7 and 10 for all drinking water samples were minor (Gillespie et al., 2014).

Figure 9 shows:

- (A) Changes of TCC on a daily basis for ten days.
- (B) Changes of ICC on an everyday basis for ten days.
- (C) Fold increase in ICC on day 7 and day 10.
- (D) Changes of TOC, TON, NH_4^+ (ammonium) and PO_4^{3-} (phosphate) concentrations on days 0, 7 and 10 (Gillespie et al., 2014).

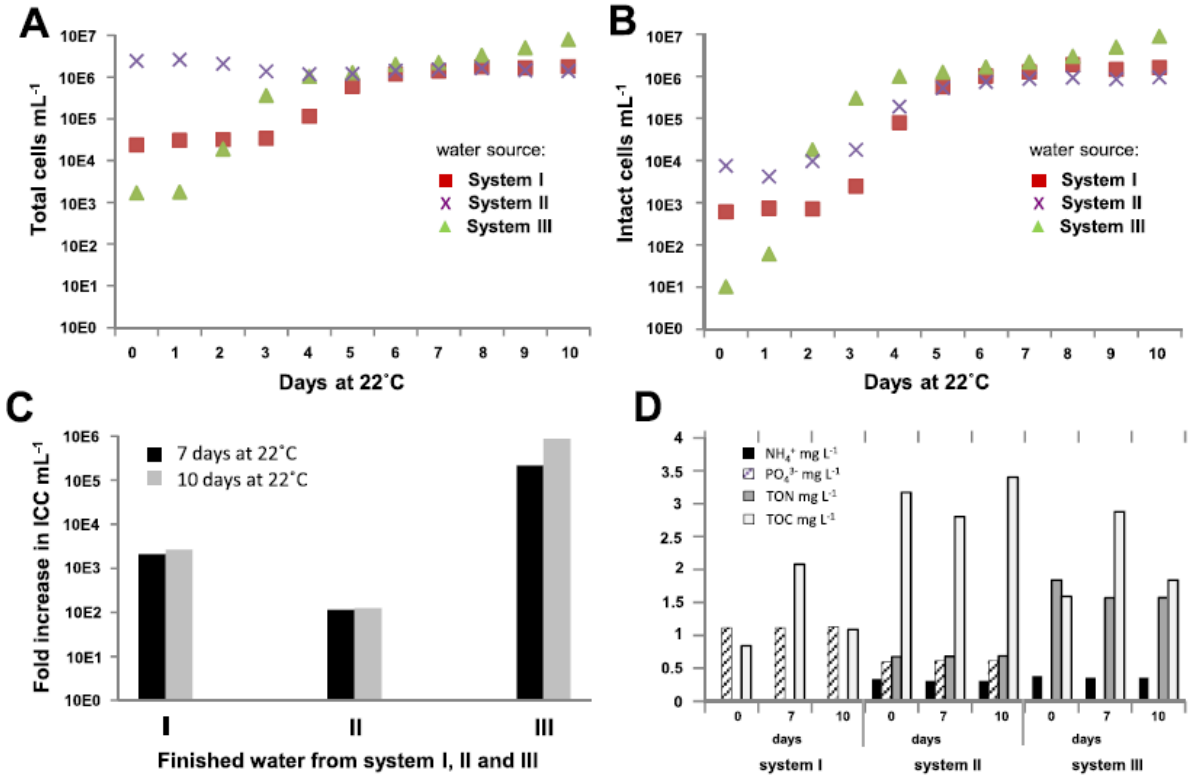

Figure 9: Bacterial growth potential in drinking water samples (Gillespie et al., 2014)

In conclusion Gillespie et al. (2014) intended to use flow cytometry in analysing drinking water at waterworks and water distribution systems. More data should be collected and analysed for longer periods of time in order to measure bacterial growth potential in drinking water samples (Gillespie et al., 2014).

3.4 Real-time flow cytometry

Nowadays flow cytometry with fluorescence is an accurate system for the detection, registration and knowledge provision about the dynamics of cells (Amor et al., 2002).

Experiments in real-time flow cytometry were done:

- for determining the microbiology of drinking water for a period longer than one day (Hammes et al., 2012);
- with household drinking water after an overnight standstill of 14 hours in the system of pipes (Prest et al., 2013);
- for measuring bacteria in drinking and river water (Besmer et al., 2014);
- for groundwater measuring changes in water quality in a sequence (every 15 minutes for 14 days) (Besmer et al., 2016);
- for estimating total cells counts in drinking water in three waterworks in South California (Huang et al., 2016).

The researches using real-time flow cytometry aimed to:

- monitor drinking water quality and optimize methods of analysis (Huang et al., 2016);
- to check the automated sampler and to examine how the system would work with two different stains; (Hammes et al., 2012);
- to expose changes in water quality over time (Besmer et al., 2016).

Measurements have been done using different procedures:

- The FCM was automated to sample every 5 minutes for a period longer than one day. The system was tested by using different stains (SYBR Green I and propidium iodide) (Hammes et al., 2012);
- Sampling was done directly after opening the tap at intervals of 30 seconds, 1 minute and 6 hours (comparison of tap drinking water is shown in Figure 10). The flow post tap opening was constant and was approximately 180 L h^{-1} (Prest et al., 2013);
- Flow cytometer BD Accuri™ C6 was designed with an autosampler that measured water samples in a flow, every 15 minutes. Each sample was stained, incubated for 10 minutes at 40 °C and measured. Cleaning of the system (flow cytometer) was performed every 24 hours with hypochlorite and purified water (Besmer et al., 2014);
- The samples were obtained from the groundwater well. Before distribution to customers, water was treated with UV-disinfection equipment. Sampling was done every 15 minutes for 14 days. 1314 samples were received and analysed with the flow cytometer BD Accuri™ C6. Every 15 minutes drinking water was directed to the autosampler and measured. All samples were stained with SYBR Green I, incubated 10 minutes at 37 °C and measured. Cleaning of the equipment with hypochlorite solution was done daily (Besmer et al., 2016).
- Before measurements were performed, 1 ml of sample was treated with 3% glutaraldehyde for 15 minutes at 4 °C. Total cell count was measured with diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} dilutions) samples. Staining was done with SYBR Gold with incubation time of 15 minutes (Huang et al., 2016).

The results of all the mentioned researches showed:

1. Prest et al. (2013) noted that the first water sample, which was taken directly after tap opening carried a significantly higher cell concentration than the subsequent ones. The cell concentration decreased notably after 30 seconds of flushing and reduced continuously after 1 hour (200 L) of flushing (shown in Figure 10). Subsequently the variation of cell concentrations was smaller than 2%. The enormous growth in cell concentrations after the standstill of drinking water in the system of pipes overnight was

linked to either bacterial regrowth or separation of the biofilm from the pipe surfaces or both of these effects (Prest et al., 2013).

Figure 10 shows:

- (A) Events obtained directly after tap opening and after flushing of 1.5 L and more.
- (B) Results received after flushing of 1.5, 90, 360 and 1080 L.
- (C) Shifts in cell concentrations depending on flashed water volume.
- (D) Percentage of HNA cells depending on flashed water volume (Prest et al., 2013).

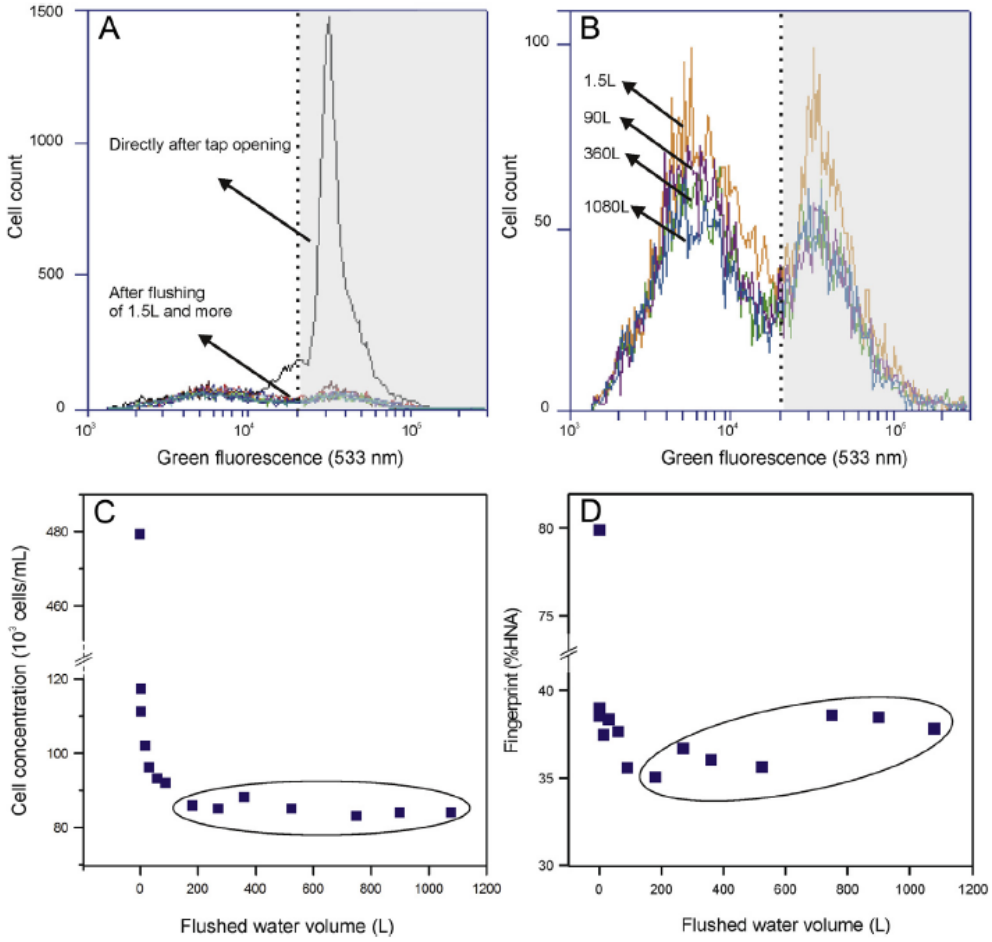

Figure 10: Comparison of tap drinking water after night standstill in various periods of time (Prest et al., 2013)

2. Automated sampler provided the possibility to get results in real-time and in 5 minute intervals. The error rate was 5% (Hammes et al., 2012).
3. Besmer et al.'s (2014) results showed that the TCC during night-time (20:00 – 08:00) increased slightly. The most considerable amount of bacteria (TCC) was measured in a period from 08:00 till 10:00. A significant decrease of TCC was observed between 10:00 – 12:00 o'clock. Significant changes in the number of measured bacteria were noticed between 12:00 – 14:00 o'clock. Moderate concentrations of cells were observed from 15:00 o'clock. The rate of LNA cells was lower at night-time in comparison to daytime (Besmer et al., 2014).
4. Besmer et al. (2016) mentioned that the number of bacteria changed during the whole time because of precipitations and changes in water consumption during day and night time. During the experiment, waterwork pumps were stopped mostly at night period

from 23:00 till 8:00. This explains the fluctuations in TTC. Another factor of changes in TCC was water precipitations. This study revealed that precipitation events influenced water quality not only in TCC but also in percentage of LNA (Besmer et al., 2016).

5. The fluctuations of detected cells in different seasons were insignificant and less than 5% (Huang et al., 2016).

Further research using real-time flow cytometry should be done:

- for checking the efficiency of FCM in the field (at the waterwork) (Hammes et al., 2012);
- in the improvement of the automated sampler (Huang et al., 2016);

Prest et al., (2013) concluded that FCM has the potential to be involved in the on-line detection and monitoring of bacterial water quality in drinking water distribution networks.

4 Material and methods

Essential materials and methods for test and research mode are defined in this part of the master thesis. Liquids, stains and settings taken as a basis for this research are also represented in this part. Settings imply incubation time for a sample measurement according to stain used. A measurement of the sample, cleaning and calibration settings are also listed in this chapter.

4.1 Equipment assembling and construction review

Online Bacteria Analyzer (OBA) has been used for testing water samples in stand trial and real-time experiment. This device worked 24 hours a day, seven days a week, only with stops for system cleaning, stain changing and other breaks for maintenance.

The general construction of OBA consists of:

1. Sample set;
2. OBA;
3. Cleaning solutions;
4. Uninterruptible Power Supply (UPS);
5. Computer facilities.

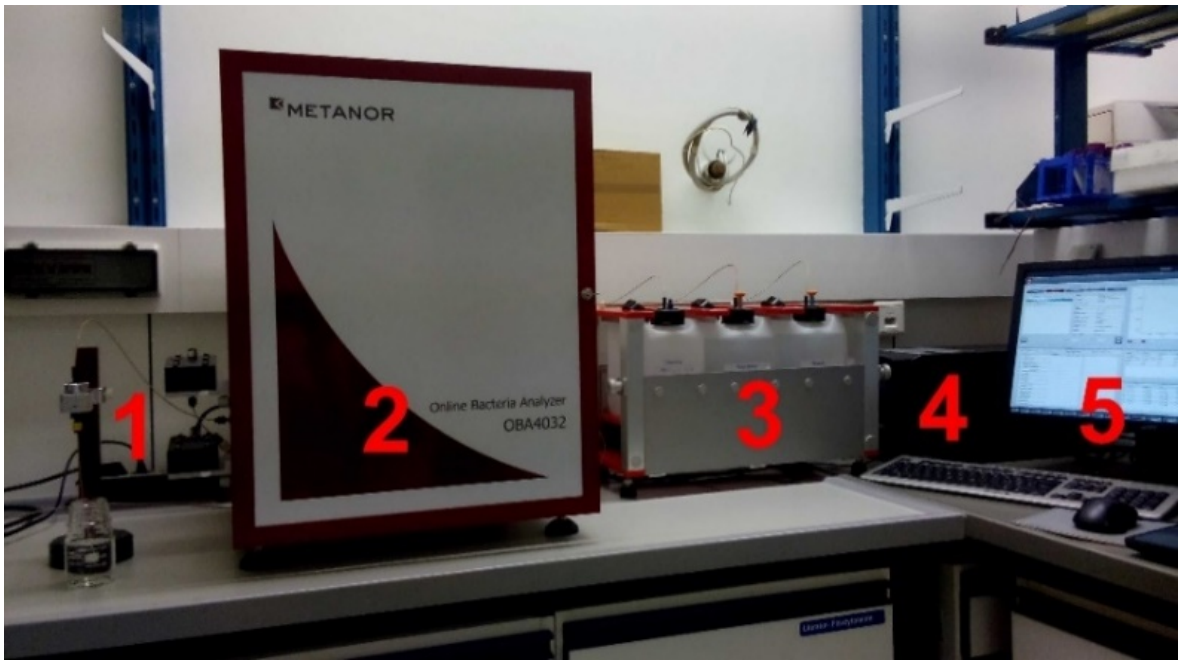

Figure 11: General construction of OBA

Sample set construction of OBA consists of:

1. Stand;
2. Coarse filter;
3. Sample Port A;
4. Sample Port B;
5. Sample.

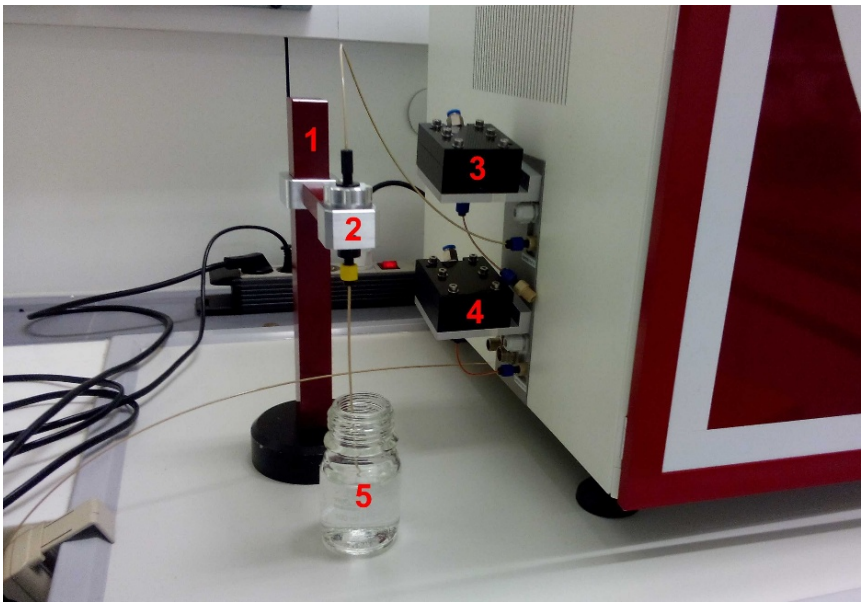

Figure 12: Sample set construction of OBA

The OBA connection pattern has the following connections to:

1. Pure water;
2. Sheath solution;
3. Cleaning solution;
4. Waste;
5. UPS-Cable;
6. Breaker (ON/OFF);
7. Monitor-Cable;
8. LAN-Cable;
9. Keyboard-Cable;
10. Mouse-Cable;
11. USB flash drive.

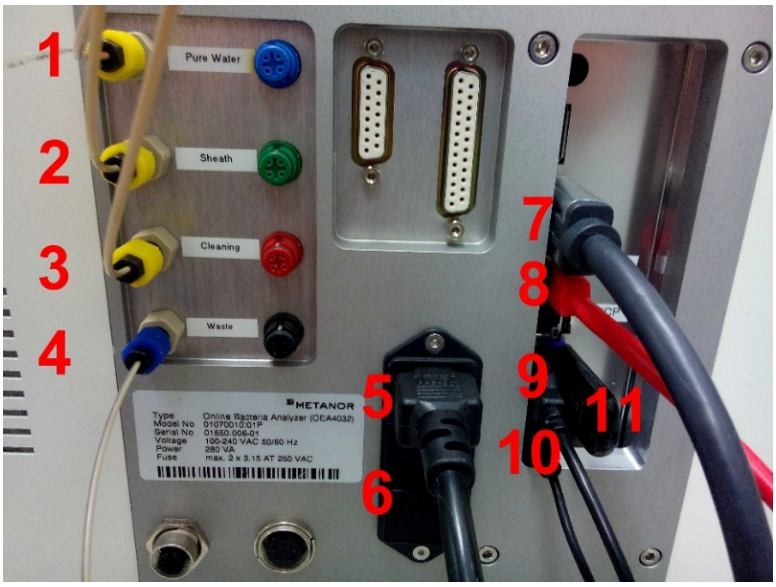

Figure 13: OBA connection pattern

Inside OBA consists of such main components:

1. Detection Unit (Photomultiplier PMT);
2. Pump units;
3. Mixer-incubator;
4. Single Port;
5. Stain unit.

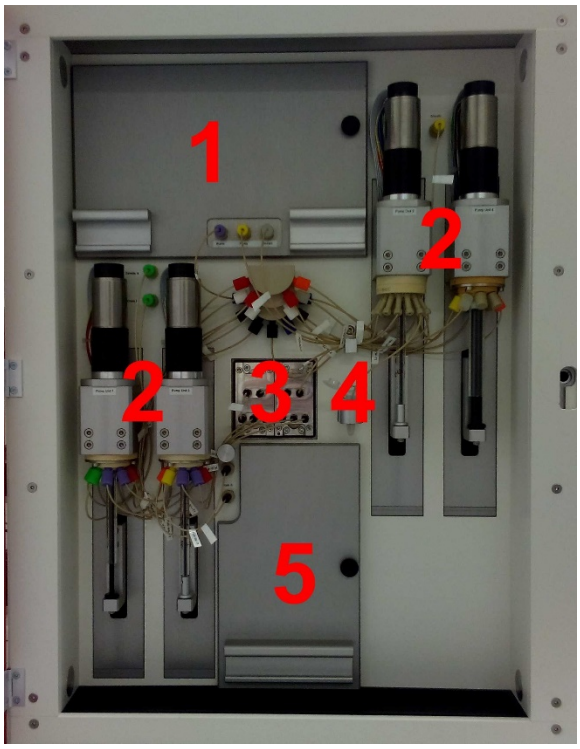

Figure 14: OBA inboard components

For precise measuring and system cleaning OBA requires liquids such as:

1. Cleaning solution (Canister 2.5 L);
2. Ultrapure water (Canister 5.0 L);
3. Sheath fluid or ultrapure water (Canister 5.0 L).

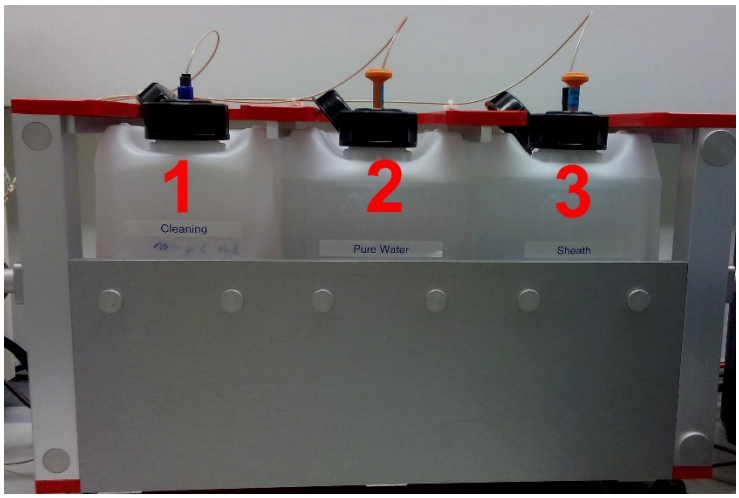

Figure 15: OBA liquid containers

After all the connections are checked and liquid containers filled, the system was activated using the procedure listed in 9.1.1 Activation.

4.1.1 Preparation of fluids and stains

For reliable measurements and system cleaning by OBA liquids such as pure water, sheath solution and cleaning solution were prepared after a defined procedure.

Pure water and sheath solution were prepared in this order:

1. Pure water from arium®pro ultrapure water systems equipment was taken in sterilised bottle;
2. Vacuum filtration devices VacuCap® pore size 0.1 µm, filter size 60 mm was taken;
3. New sterilised bottle was taken, and filter was placed on it;
4. Vacuum pump was connected to pure water filtration;
5. Filtration started;
6. After filtration pure water/sheath was filled in OBA liquid containers (see Figure 15: OBA liquid containers);
7. Level of OBA liquid containers was set in software.

The cleaning solution was prepared as such:

1. Prepared pure water for OBA was taken as basis for solution;
2. Afterwards, sodium hypochlorite was diluted with pure water for OBA to a final concentration 0.1%;
3. Level of OBA liquid containers was set in software.

OBA has had two stain units that were located on the equipment (see Figure 14: OBA inboard components).

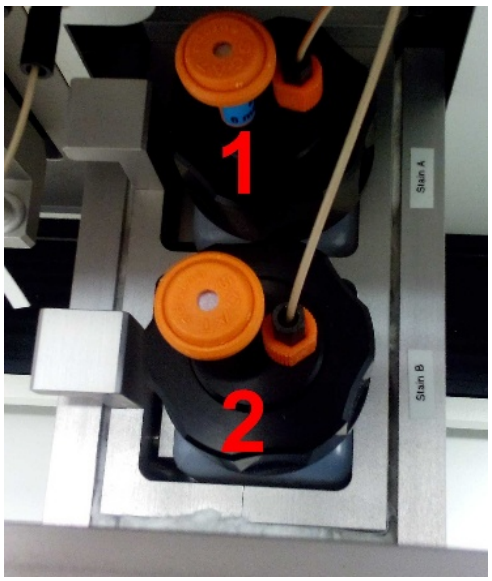

Figure 16: Stain units

Stain preparation procedure was done carefully and with labour protection clothes. For stain preparation, these facilities were needed:

1. Plastic OBA container (100 ml);
2. Syringe (10 or 20 ml);
3. Plastic tube (50 ml);
4. Syringe filter 0.1 µm.

Figure 17: Facilities for stain preparation

Preparation steps done for stain A and B are listed in the Table 1 and Table 2.

SYBR Green prepared by METANOR© were also used in test mode. Preparation steps and composition are listed in the Table 3.

Stain usability was no longer than six months and stored at 15 °C. The storage of prepared stains at 15 °C was either located in the stain units or in the laboratory incubator. For changing stain in the system, see chapter 9.1.2 Stain change application.

Table 1: Stain A (SYBR Green propidium iodide) preparation steps

Preparation steps	**for 50 ml of stain, µl**	**for 20 ml of stain, µl**
1. Components blended in 50 ml plastic (PP) tube		
SYBR Green (stored at -20°C)	100	40
TRIS-HCI solution 10 mM (pH=8)	500	200
Pure water	47400	18960
Solution of propidium iodide (1 mg/ml)	2000	800
2. Blend filtered with 0.1 µm filter membrane		
3. Filtered blend poured into 100 ml plastic OBA container		

Table 2: Stain B (SYBR Green) preparation steps

Preparation steps	**for 50 ml of stain, µl**	**for 20 ml of stain, µl**
1. Components blended in 50 ml plastic (PP) tube		
SYBR Green (stored at -20°C)	100	40
TRIS-HCI solution 10 mM (pH=8)	500	200
Pure water	49400	19760
2. Blend filtered with 0.1 µm filter membrane		
3. Filtered blend poured into 100 ml plastic OBA container		

Table 3: Stain C (SYBR Green by METANOR©) preparation steps

Preparation steps	for 50 ml of stain, µl	for 20 ml of stain, µl
1. Components blended in 50 ml plastic (PP) tube		
SYBR Green (stored at -20°C)	100	40
Dimethyl Sulfoxide (DMSO)	9900	3960
TRIS-HCI solution 1 M (pH=8)	5	2
Sodium acid	500	200
Pure water	39495	15798
2. Blend filtered with 0.1 µm filter membrane		
3. Filtered blend poured into 100 ml plastic OBA container		

4.1.2 Cleaning of the equipment

The cleaning of OBA was performed after making a set of measurements (≈10 - 20 analysis). Cleaning was done with cleaning solution and pure water. After being cleaned with “cleaning solution”, that contained sodium hypochlorite, the system was rinsed with pure water. This step was necessary to remove traces of cleaning solution that could have interfered with the measurements. Pure water cleaning was done by using a little amount of cleaning solution and a massive amount of pure water. Remarks, current problems and recommendations by cleaning application are listed in 9.1.3 Cleaning application.

Pure water quality is essential for sample analysis. Canister with pure water was checked. For information on the procedure for pure water canister measurement see chapter 9.1.4 Measure application.

4.1.3 Liquid consumption per process/per week

Information about the level of liquid containers is listed in administration menu, subsection settings. Settings show the current volume of liquid containers.

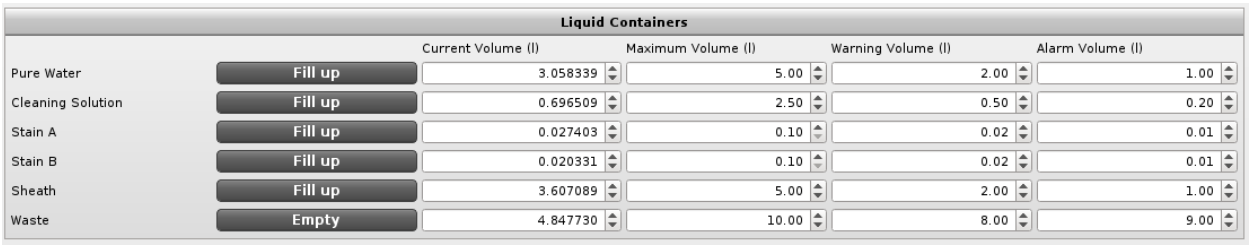

Figure 18: Liquid containers settings

Current volume (in liters) was input manually into the system which then performed calculations automatically.

Warning and alarm volume were defined by the user. When the current volume of some liquid reached warning or alarm volume, the user received an e-mail that the liquid container should be filled up.

Average consumption per process was counted after series of measurements. “Consumption per process” meant consumption of liquids after measuring the sample with sample port A or B. Sample port A or B needed an average sample volume of 3 ml. In Table 4: Average consumption of liquids per process when only one stain (stain A or stain B) was consumed.

Table 4: Average consumption of liquids per process

Liquid	**Consumption per process [L]**
Pure water	0.054565
Cleaning solution	0.000681
Stain A (SGPI)	0.000060
Stain B (SG)	0.000073
Sheath solution	0.003499
Waste	0.010953

Average consumption of liquids per week when OBA was working 24 hours a day, seven days a week is presented in the table below.

Table 5: Average consumption of liquids per week

Liquid	**Consumption per week [L]**
Pure water	2.0
Cleaning solution	0.03
Stain A (SGPI)	0.015
Stain B (SG)	0.015
Sheath solution	2.0
Waste	5.5

Liquid containers were refilled with defined liquids. Only waste container with discharge liquid was afterwards emptied and utilised.

Pure water, sheath solution and stains were prepared every week freshly.

4.1.4 Statement of process and sequence

The analysis of water samples from the beginning until the end was named process. The process is further divided into three main parts: sample preparation, sample measurement and analysis. The block diagram for process is represented below.

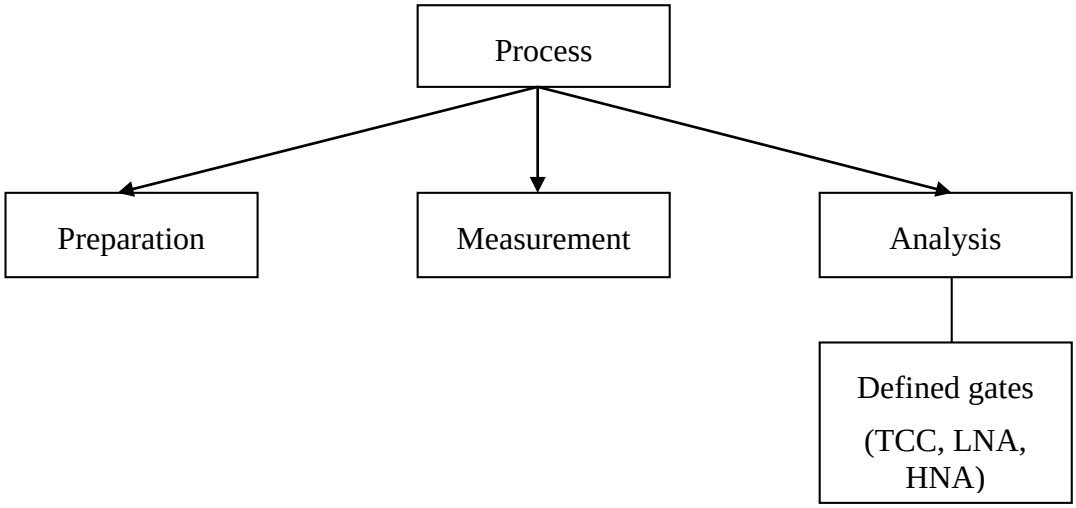

Figure 19: Diagram of process

Sample preparation included:

- Sample port A, sample port B or single port selection;
- Stain A or Stain B selection.

Sample measurement could be selected from templates such as: Regular, Regular_2000 or Regular_5000. The difference between these templates was in the minimum size of the detected trigger.

Sample analysis was based on measuring plot area (Gate) that included information about TCC, LNA and HNA (see chapter 4.1.5 Measuring plot area (Gating)).

The sequence in the experimental case is defined as a process/processes that was/were repeated several times at fixed time intervals. The sequence consists of process, system cleaning and meta-analysis. Block diagram for sequence is represented below.

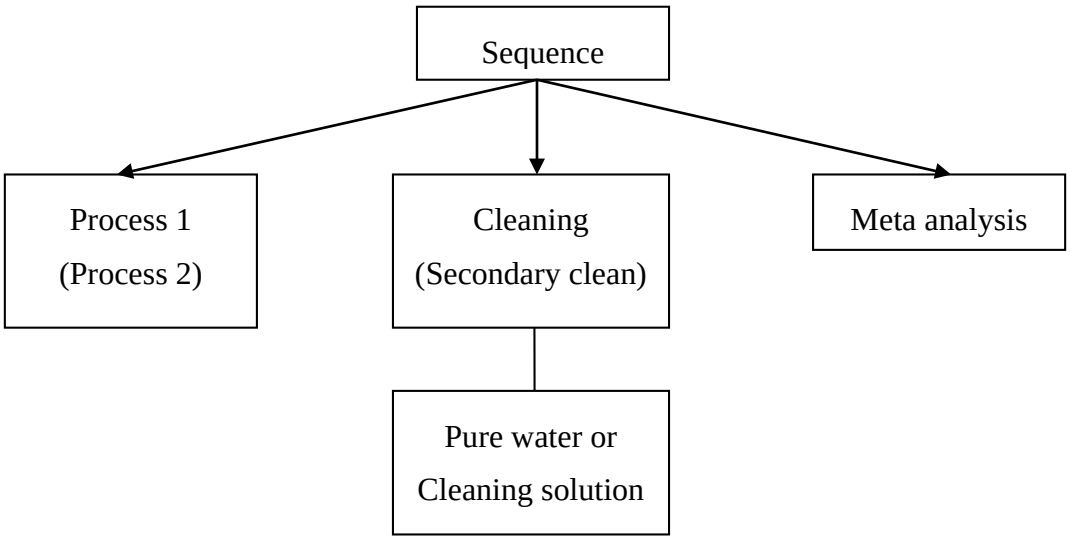

Figure 20: Diagram of sequence

Process 1 and Process 2 could be selected from defined templates. At least one process was selected. For information about sequence settings see chapter 9.1.6 Sequence application.

System cleaning by OBA consists of clean and secondary clean. At least one of these options should be selected. Clean could be selected between pure water or cleaning solution with defined intervals. In the research pure water cleaning was employed. Secondary clean was added only when the system had been working without stopping for more than seven days. For secondary clean, cleaning solution was used, for ensuring cleaner conditions for subsequnt measurements. For information about cleaning see chapter 4.1.2 Cleaning.

Meta-analysis was an option and was used for making calculations that were based on one or two previously defined analysis. At least one defined analysis and measuring plot area (gate) should be selected. For meta-analysis a gate, described in chapter 4.1.5 Measuring plot area (Gating) was used.

4.1.5 Measuring plot area (Gating)

4.1.5.1 Measuring plot area (Gate) in lab scale research

The measurements were performed with 8-peak calibration beads for the set of the measuring plot area (gate). The gate provides information about the total cell count (TCC), low nucleic acid cells (LNA) and high nucleic acid cells (HNA). Not only OBA but also BD Accuri™ C6 Plus experimented with calibration beads. This experiment was done for setting the gate for OBA in the same plot area like in BD Accuri™ C6 Plus. To have the same measuring plot area was necessary for comparing results of subsequent measurements.

Measuring plot area by OBA is represented below.

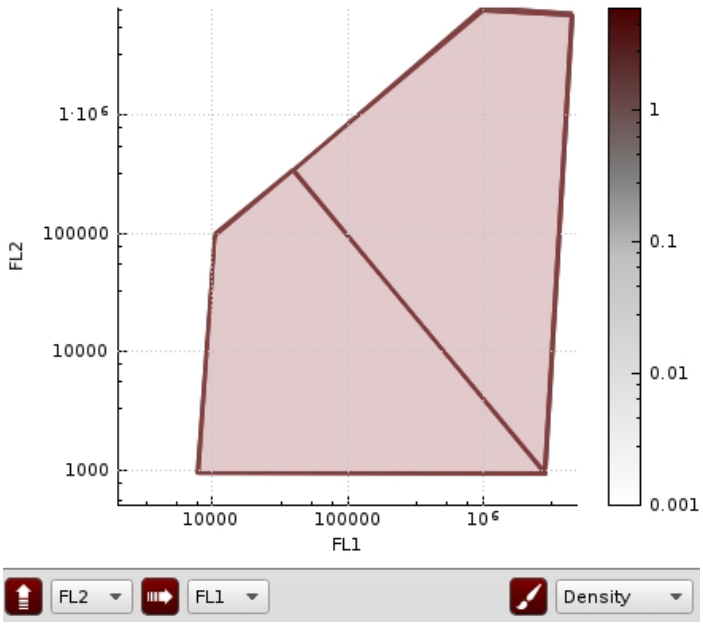

Figure 21: Measuring plot area (Gate) by OBA

Measuring plot area for TCC, LNA and HNA in lab scale (listed below) has also been used by experiments such as those listed in chapter 5.1.1 Stain and incubation times, chapter 5.1.4 Effect of system cleaning, chapter 5.1.5 Test mode stand trial experiment and chapter 5.1.6 Test mode real-time experiment.

Table 6: Measuring plot area points for TCC, LNA and HNA in lab scale

Gate Points	TCC		LNA		HNA	
	x	y	x	y	x	y
1	7885	938	33700	260000	33700	260000
2	12200	95128	12200	95128	2931840	938
3	1008050	7635350	7885	938	4663710	6955170
4	4663710	6955170	2931840	938	1008050	7635350
5	2931840	938	33700	260000	337000	260000
6	7885	938	-	-	-	-

Measuring plot areas TCC, HNA and LNA for analysis were gathered in one gate named BOKU_01.

4.1.5.2 Measuring plot area (Gate) in field research

For field experiments another measuring plot area for TCC, LNA and HNA was used. Depending on the stain, different fixed gates were used. For SYBR Green stain in chapter 5.2.1 Research mode stand trial experiment gate points are listed below.

Table 7: Gate points for SYBR Green stain in research mode by stand trial experiment

Gate Points	TCC		LNA		HNA	
	x	y	x	y	x	y
1	7885	938	22000	270000	22000	270000
2	12200	160000	12200	160000	2931840	938
3	1008050	7635350	7885	938	4663710	6955170
4	4663710	6955170	2931840	938	1008050	7635350
5	2931840	938	22000	270000	22000	270000
6	7885	938	-	-	-	-

For SGPI stain in chapter 5.2.1 Research mode stand trial experiment used gate points are listed below.

Table 8: Gate points for SGPI stain in research mode by stand trial experiment

Gate Points	TCC		LNA		HNA	
	x	y	x	y	x	Y
1	7885	938	22000	500000	22000	500000
2	13000	330000	13000	330000	2931840	938
3	1008050	7635350	7885	938	4663710	6955170
4	4663710	6955170	2931840	938	1008050	7635350
5	2931840	938	22000	500000	22000	500000
6	7885	938	-	-	-	-

For SYBR Green stain in (5.2.2 Research mode real-time experiments), gate points, the same measuring plot area as that listed in (Table 7: Gate points for SYBR Green stain in research mode by stand trial experiment) was used.

For SGPI stain in (5.2.2 Research mode real-time experiments) used gate points listed below.

Table 9: Gate points for SGPI stain in research mode by real-time experiment

Gate Points	TCC		LNA		HNA	
	x	y	x	y	x	y
1	7885	938	25000	600000	25000	600000
2	10500	330000	10500	330000	2931840	938
3	1008050	7635350	7885	938	4663710	6955170
4	4663710	6955170	2931840	938	1008050	7635350
5	2931840	938	25000	600000	25000	600000
6	7885	938	-	-	-	-

Different gates were used because of various water samples.

4.2 Laboratory experiment

4.2.1 Stain and incubation times

4.2.1.1 Stain and incubation times TCC

Stain difference was the first point in the research. The aim of this analysis was to move away from METANOR© produced stains and applying the self-prepared stains at BOKU. This step was important for having freshly prepared stains that were used in next water analysis. Adjustment of the incubation time of staining was aimed to detect all microbial cells in water and to obtain good separation of bacterial cells.

Measurements were performed with different stains:

- Stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2);
- Stain C – SYBR Green prepared by METANOR© (for preparation steps and composition see Table 3).

Measurements were performed by different incubation times: 3 min 20 sec; 4 min; 5 min; 8 min. Incubation by OBA was provided internally. The incubation temperature of staining for all water samples was the same and fixed at 40°C. This feature was the reason for checking different incubation times.

Applied settings to sample measurements for different stains are listed in the Table 10.

The list of provided experiments with different prepared stains are listed in the Table 11.

Table 10: Measure settings for differences between stains experiment

Name	Value
Measure time	150 sec
Stain/Sample ratio	1:9
Incubation temperature	40 °C
Trigger method	OBA SmartDetect™
Minimum size	5000
Minimum length	5

Table 11: List of the stain and incubation time differences experiments

Name	Sample	Stain and incubation time	Cleaning
Sample 3	Well water	Stain B : 8 min ; Stain C : 3 min 20 sec.	No cleaning
Sample 4	Tap water	Stain B : 8 min ; Stain C : 3 min 20 sec.	No cleaning
Sample 5	Tap water	Stain B : 8 min ; Stain C : 3 min 20 sec.	No cleaning
Sample 6	Well water	Stain B : 4 min ; Stain C : 3 min 20 sec.	No cleaning
Sample 7	Well water	Stain B : 5 min ; Stain C : 5 min .	No cleaning

4.2.1.2 Stain and incubation times ICC

The other stain SYBR Green propidium iodide was examined by different incubation times. Adjustment of the incubation time of staining was aimed to detect all microbial cells in water and to obtain good separation of bacterial cells. Incubation by OBA was provided internally. This feature was the reason for checking different incubation times.

Measurements were performed with Sample Port A with different stains:

- Stain A – SYBR Green propidium iodide prepared at BOKU (for preparation steps and composition see Table 1).

Measurements were performed by different incubation times: 5 min; 10 min.

Applied settings to sample measurements for SYBR Green propidium iodide stain are listed in the Table 10.

4.2.2 Stability of stains

The analyses are aimed to check the stability of SG and SGPI stains.

Measurements were performed with two different stains stored at 15 °C:

- Stain A – SYBR Green propidium iodide prepared at BOKU (for preparation steps and composition see Table 1);
- Stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2).

Measurements were performed by two incubation times:

- Stain A by 10 min;
- Stain B by 5 min.

For examination old stains made on 09.06.2017 were used and compared to stains prepared on:

- 21.08.2017 (time difference two months);
- 09.10.2017 (time difference four months).

Measurements were performed with BD Accuri™ C6 Plus. The list of provided experiments for checking the stability of stains are listed in the Table 12.

Table 12: List of the stability of stains experiments

Name	Sample	Stain and incubation time	Cleaning
21.08.2017	Well water	Stain A : 10 min ; Stain B : 5 min .	No cleaning
09.10.2017	Well water	Stain A : 10 min ; Stain B : 5 min .	No cleaning

4.2.3 Calibration measurements

Calibration measurements are aimed to check OBA flow-cytometer on the stability of measuring. The measurements were performed with one peak calibration beads. Beads measurements were done by using a single port. Steps are listed in 9.1.5 Calibration application. Gate points for one peak beads are presented in Table 13.

Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus and CyFlow® Cube 6. Obtained results were compared between all applied flow cytometers.

Table 13: Beads measuring plot area points

№	Gate points	
	x	y
1	3975960	3855830
2	4104150	11952000
3	8206310	15903700
4	12116200	17927800
5	15961900	188843400
6	19423100	18361500
7	19935900	15951900
8	20000000	12000200
9	15385100	8096680
10	11667500	5638910
11	8206310	4144970
12	3975960	3855830

4.2.4 Effect of system cleaning

4.2.4.1 Effect of system cleaning with pure water

Cleaning of the system was a point of discussion because OBA, by sample port cleaning pulled pure water in the sample. The amount of this pull was about 0,5 ml per one system clean if the setting was done with “Pure Water cleaning with one operation count”. For technical details about pure water cleaning see chapter 9.1.3 Cleaning application.

The pure water was sprayed in to the sample port either by “pure water” or by “cleaning solution” application. For checking the possibility of influence of pulls with pure water on the sample, measurements were performed with Stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2). The incubation time was set at 5 minutes. For water sample was used: UV-C processed water. The cleaning was done after 5 hours of measurements. Applied settings to sample measurements for stain B are listed in the Table 14.

4.2.4.2 Effect of system cleaning with cleaning solution

This analysis was aimed to check the influence of cleaning solution application (contained sodium hypochlorite) on the obtained results. The following analysis was carried out every hour. The incubation time was set at 5 min for Stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2). The water sample that was used: untreated raw water. The cleaning was done after 10 hours of measurements. Applied settings to sample measurements for stain B are listed in the Table 14.

4.2.5 Test mode stand trial experiment

The stand trial was aimed to analyse the growth of bacteria in raw water. For stand trial experiment muffled 250 ml bottles wrapped in aluminium foil were chosen.

All preparation procedures for scientific experiments were done under sterile conditions. For stand trial experiments the following were used:

- Sterilized (muffled) bottles 250 ml;
- Magnet, sterilized with cleaning solution and pure water;
- Magnetic sitter;
- Aluminium foil for wrapping muffled 250 ml bottles.

After preparation, the sample was measured with sample port A.

Measurements were performed with Sample Port A with different stains:

- Stain A – SYBR Green propidium iodide prepared at BOKU (for preparation steps and composition see Table 1);
- Stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2).

Measurements were performed by different incubation times:

- Stain A: 10 min;
- Stain B: 5 min.

During the stand trial experiment, there was no cleaning.

Applied settings to sample measurements for different stains are listed in the table below.

Table 14: Measure settings for test mode by stand trial experiment

Name	Value
Measure time	150 sec
Stain/Sample ratio	1:9
Incubation temperature	40 °C
Trigger method	OBA SmartDetect™
Minimum size	5000
Minimum length	5

4.2.6 Test mode real-time experiment

The real-time experiment was aimed to monitor microbiological changes in tap water with continuous sampling every 2 hours.

All preparation procedures for the experiment were done under sterile conditions. For real-time experiment these were used:

- Autoclaved PTFE hose pipe for input;
- Autoclaved PTFE hose pipe for output;
- Water-flow at the rate of 25 litres per hour.

Technical specifications for input and output PTFE hose pipes are listed below:

Table 15: Technical specifications for PTFE hose pipes

Name	**Characteristic**
Material	Polytetrafluoroethylene (PTFE)
ID (inside diameter)	6 mm
OD (outside diameter)	8 mm
Operating pressure	15 bar
Radius of bend	68 mm
Density	2.14 – 2.20 g/cm³
Color	Nature
Safety factor	3:1
Temperature range	-75 °C to +260 °C

PTFE Hose pipes were autoclaved under settings listed below:

Table 16: Autoclave settings for PTFE hose pipes

Name	**Setting**
Autoclave	Systec V-Series
Application	Sterilization of solids
Sterilization temperature	121 °C
Sterilization time	20 min
Dry time	10 min
End temperature	120 °C

Preparation procedure by real-time experiment was done in the following range:

1. Autoclaved hose pipe for input was connected from water flow to sample port;
2. Autoclaved hose pipe for output was connected from sample port to outflow;
3. Water-flow was progressively set at the rate of 25 litres per hour.

Connection schema by real-time experiment is depicted below.

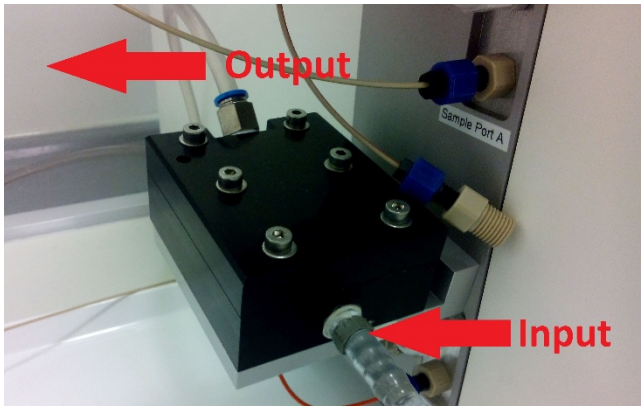

Figure 22: Connection schema by real-time experiment

Measurements were performed with Sample Port B with two stains:

- Stain A – SYBR Green propidium iodide prepared at BOKU (for preparation steps and composition see Table 1);
- Stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2).

Measurements were performed by two incubation times:

- Stain A by 10 min;
- Stain B by 5 min.

During the real-time experiment, there was no cleaning. The steps of one measuring process are listed in chapter 9.1.4 Measure application. The steps for two processes done at set time intervals are listed in chapter 9.1.6 Sequence application. Settings applied to sample measurements for two stains are listed in the Table 17.

Table 17: Settings for test mode by real-time experiments

Name	Value
Measure time	150 sec
Stain/Sample ratio	1:9
Incubation temperature	40 °C
Trigger method	OBA SmartDetect™
Minimum size	5000
Minimum length	5

4.3 Field experiment

4.3.1 Research mode stand trial experiment

In research mode by stand trial experiment the following were used:

- Muffled 2 L bottles wrapped in aluminium foil;
- Incubator with set temperature at 15 °C;
- Drinking water after UV-C treatment.

Preparation procedure started with a collection of water samples directly from waterworks in muffled 2 L bottles wrapped in aluminium foil. Afterwards, sample and reference sample were placed in an incubator with the set temperature at 15 °C. Measurements were started when the temperature of samples reached 15 °C. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus.

Incubator's temperature for experiments is listed below.

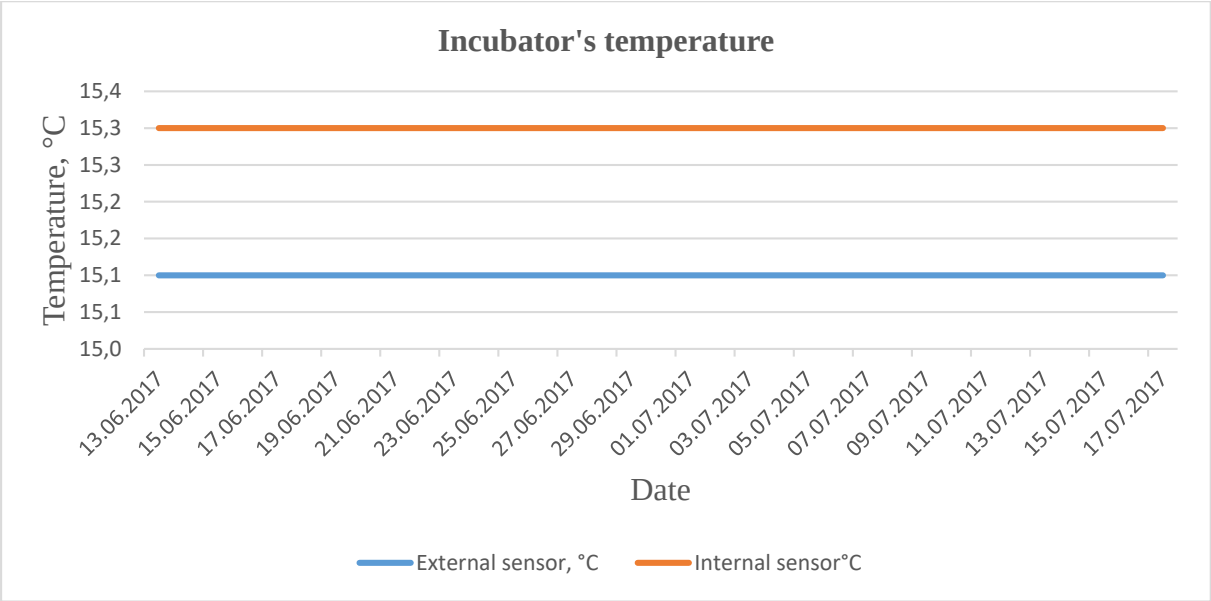

Figure 23: Incubator's temperature by stand trial experiment

After preparation the sample was measured with sample port A.

The experiment was done with two stains:

- Stain A – SYBR Green propidium iodide prepared at BOKU (for preparation steps and composition see Table 1);
- Stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2).

Measurements were performed by two incubation times:

- Stain A by 10 min;
- Stain B by 5 min.

Settings applied to sample measurements for two stains are listed in the table below.

Table 18: Settings for research mode by stand trial experiment

Name	Value
Measure time	150 sec
Stain/Sample ratio	1:9
Incubation temperature	40 °C
Trigger method	OBA SmartDetect™
Minimum size	5000
Minimum length	5

For information on measuring plot area for experiment see Table 6: Measuring plot area points for TCC, LNA and HNA .

The duration of each stand trial experiment was seven days. This time interval was chosen for exploring growth of cells in drinking water, because in normal cases the distribution of water to the consumer is carried out during this period of time. Measurements were started when water sample reached 15 °C. There was no cleaning during sample measuring.

4.3.2 Research mode real-time experiment

Untreated raw water was analysed from the flow during the real-time experiment in the research mode. The experiment was done 24 hours a day, seven days a week (24/7) for observing changes in the flow of untreated raw water. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus.

Preparation procedure, connection schema and measurements were done under the same conditions that are described in 4.2.6 Test mode real-time experiment.

OBA was installed at the waterwork and connected to a raw water sampler (shown below).

Figure 24: Installed OBA at waterwork

Cleaning was done only once a week with cleaning solution and pure water. Cleaning procedure is listed in chapter 9.1.3 Cleaning application.

4.3.3 List of field research experiments

Table 19: List of the field research experiments

Name	Sample	Stain and incubation time	Cleaning
Experiment type: stand trial			
13.06.2017 – 20.06.2017	UV-C processed water (15 °C)	Stain A : 10 min ; Stain B : 5 min .	No cleaning
20.06.2017 – 27.06.2017	UV-C processed water (15 °C)	Stain A : 10 min ; Stain B : 5 min .	No cleaning
03.07.2017 – 11.07.2017	UV-C processed water (15 °C)	Stain A : 10 min ; Stain B : 5 min .	No cleaning
11.07.2017 – 17.07.2017	UV-C processed water (15 °C)	Stain A : 10 min ; Stain B : 5 min .	No cleaning
05.09.2017 – 11.09.2017	UV-C processed water (15 °C)	Stain B : 5 min .	Every 24 hours with pure water
26.09.2017 – 10.10.2017	Treated water with 0.1 mg/L ClO_2 (15 °C)	Stain B : 5 min .	Every 24 hours with pure water
Experiment type: real-time experiment			
03.07.2017 – 11.07.2017	Untreated raw water	Stain A : 10 min ; Stain B : 5 min .	No cleaning
11.07.2017 – 17.07.2017	Untreated raw water	Stain A : 10 min ; Stain B : 5 min .	No cleaning
17.07.2017 – 25.07.2017	Untreated raw water	Stain A : 10 min ; Stain B : 5 min .	Every 24 hours with cleaning solution
25.07.2017 – 01.08.2017	Untreated raw water	Stain A : 10 min ; Stain B : 5 min .	Every 12 hours with cleaning solution
01.08.2017 – 08.08.2017	Untreated raw water	Stain A : 10 min ; Stain B : 5 min .	Every 12 hours with cleaning solution
08.08.2017 – 17.08.2017	Untreated raw water	Stain A : 10 min ; Stain B : 5 min .	Every 16 hours with cleaning solution

4.4 Statistics

The obtained data from measurements was calculated using average and standard deviation equations. The average (mean value) is the sum of all obtained values (bacterial cells) divided by the number of all measurements. The average (mean value) formula is represented below:

$$A = \frac{1}{n}\sum_{i=1}^{n} a_i = \frac{a_1 + a_2 + \cdots + a_n}{n} \quad (1)$$

where, $a_1, a_2, \ldots, a_n$ = obtained values (bacterial cells);

n = the number of all measurements (Diggle and Chetwynd, 2011).

Standard deviation is the amount of variation of obtained data values (Bland and Altman, 1996). The formula of standard deviation is represented below:

$$s = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2} \quad (2)$$

where, $\{x_1, x_2, \ldots x_n\}$ = values of water measurements;

$\bar{x}$ = mean value of obtained results;

n = the number of samples in research (Hess and Hess, 2016).

The calculations of average and standard deviation were performed for water samples' results, obtained through lab scale (see chapter 5.1 Lab scale results) and field researches (see chapter 5.2 Field research results).

5 Results and discussion

5.1 Lab scale results

5.1.1 Stain and incubation times

The aim of stain and incubation times analysis was to move away from METANOR© produced stains and applying the self-prepared stains at BOKU. An issue regarding transfer from stain prepared by METANOR© to stain prepared at BOKU happened due to the difficulties in transportation of stain. This step was important for having freshly prepared stains that were used in field research (results are shown in chapter 5.2 Field research results).

Adjustment of the incubation time of staining was aimed to detect all microbial cells in water and for getting better separation of background. Incubation by OBA was provided internally and the incubation temperature of staining for all analyses was 40 °C. This feature was the reason for checking different incubation times. The goal was to find the optimal incubation time for stains prepared at BOKU (stain A and stain B).

5.1.1.1 Stain and incubation times for TCC determination

Stain difference was the first point in lab scale research. For analysis two different stains were used:

- Stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2: Stain B (SYBR Green) preparation steps).
- Stain C – SYBR Green prepared by METANOR© (for preparation steps and composition see Table 3: Stain C (SYBR Green by METANOR©) preparation steps);

Measurements were done by different incubation times:

- Stain B: 8 min. According to the research conducted by Van Nevel et. al. (2016) where the incubation time of 13 minutes at 37 °C for total cell count with SYBR Green I was used and due to the research done by Prest et. al. (2013) that employed an incubation time of 5 minutes at 35 °C was decided to set an average incubation time value at 8 minutes for stain B. The incubation temperature of staining for all analyses was 40 °C. The temperature of the incubation could not be changed due to the design features of the equipment.
- Stain C: 3 min 20 sec. The incubation time was recommended by the stain manufacturer.

Measurements were done every hour. Water samples used:

- Well water (Sample 3). Results are listed in Table 20.
- Tap water (Sample 4). Results are listed in Table 21.

Results shown in Table 20 and Table 21 demonstrated that the number of detected cells that where incubated for 8 minutes were 5 – 10 % higher than the sample which where incubated for 3 minutes 20 seconds. This distinction in the results and therefore this incubation time set was checked in the subsequent analysis for a more extended period of time.

Table 20: Stain difference Sample 3 results

Time, hour	Stain B, TCC/ml	Stain C, TCC/ml
0	305792	290637
1	328488	325940
2	368688	371933
3	393429	394451

Table 21: Stain difference Sample 4 results

Time, hour	Stain B, TCC/ml	Stain C, TCC/ml
0	215481	204925
1	228288	209948
2	223881	209200
3	234570	213029

The following analysis was carried out every two hours. The incubation time was not changed for checking the distinction between the results. Incubation time was adjusted for:

- Stain B by 8 min;
- Stain C by 3 min 20 sec;

Water samples used:

- Tap water (Sample 5). Results are shown in Figure 25.

Results depicted in Figure 25 show that the stained sample with stain B, which was incubated for 8 minutes, had less cells detected in comparison to stain C. The number of detected cells that where incubated for 3 minutes 20 seconds were 5 – 10 % higher than the sample which where incubated for 8 minutes. The standard deviation by stain B was 4900 count/ml and 4200 count/ml by stain C, which means that the obtained data values were approximately the same in the amount of variation. After this analysis was decided to set incubation time of 4 minutes for stain B. This decision was based on the results obtained in Table 20 and Table 21.

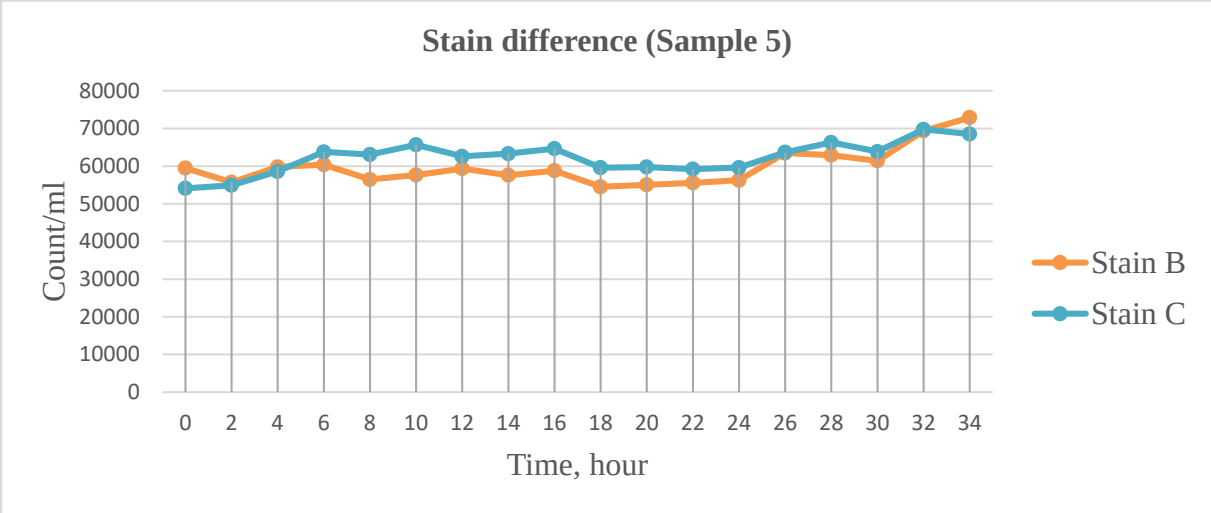

Figure 25: Stain difference Sample 5 results

Subsequent analysis was carried out every two hours. The aim was to test the distinction of detected cells with a decrease in the incubation time for stain B. Incubation time was adjusted for:

- Stain B by 4 min;
- Stain C by 3 min 20 sec.

Water samples used:

- Well water (Sample 6). Results are shown in Figure 26.

Results depicted in Figure 26 show that stain B has more cells (5 – 10 %) detected in comparison to stain C. The similar issues have been seen by analysis shown in Table 20 and Table 21. After these analyses it was concluded that lower incubation time by stain B did not dramatically change the number of detected cells, but the visual representation of results (separation of background) was sharper for longer (8 minutes) incubation time. Due to the obtained results it was decided to set the incubation time at 5 minutes for getting better separation of background.

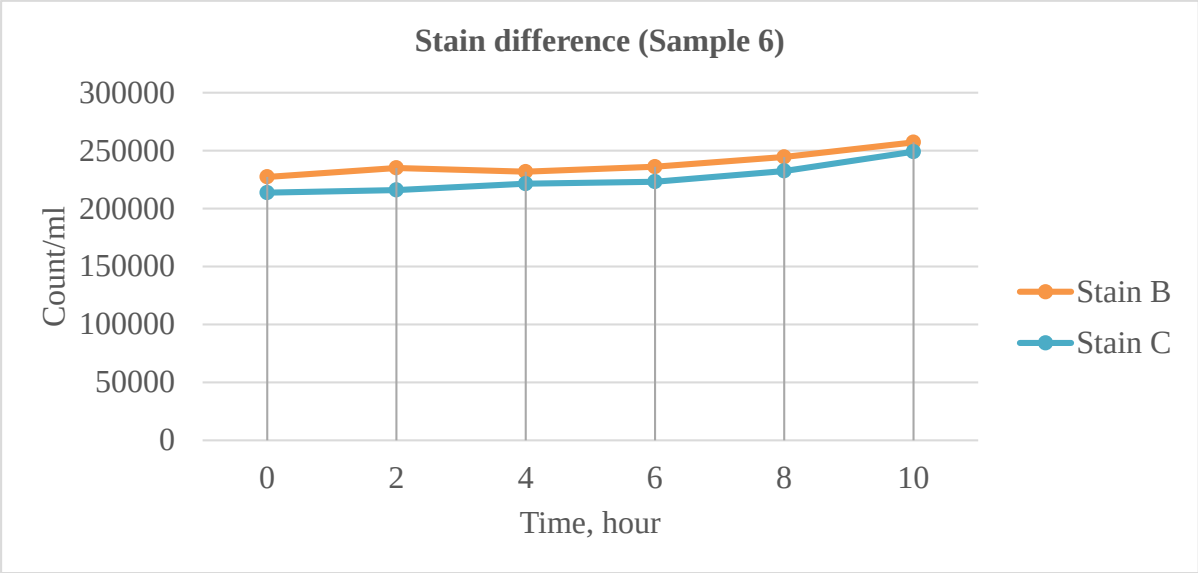

Figure 26: Stain difference Sample 6 results

According to the research conducted by Hammes et. al. (2012) where an incubation time of 5 minutes at 30 °C was used for total cell count with SYBR Green I and the obtained results from Figure 26 it was decided to set the incubation time of 5 minutes for stain B and stain C. This incubation time could be essential for better visible separation of the background. As previously mentioned, the incubation temperature for all analysis was fixed at 40 °C due to the design features of the equipment. Water samples used:

- Well water (Sample 7). Results are shown in Figure 27.

The received result depicted in Figure 27 shows that the incubation time was correctly adjusted for both stains. The distinction between received results was less than 5 %. An average of obtained cells during the whole experiment for stain B were 209000 counts/ml and 208000 counts/ml for stain C. According to the obtained results it was decided to use stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2: Stain B (SYBR Green) preparation steps) and adjust an incubation time at 5 minutes for the next experiments. Transfer from stain prepared by METANOR© to stain prepared at BOKU happened due to the difficulties in transportation of stain. The shorter incubation time was better for the next experiments that were performed in field research (results are shown in chapter 5.2 Field research results).

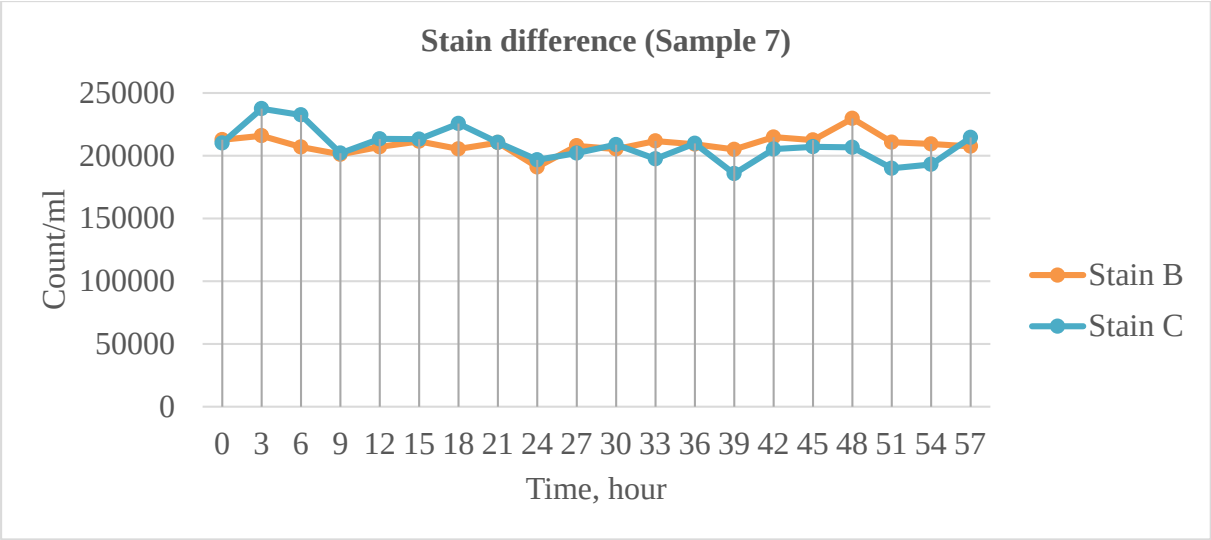

Figure 27: Stain difference Sample 7 results

5.1.1.2 Stain and incubation times for ICC determination

The other stain SYBR Green propidium iodide (for preparation procedure and composition see Table 1) was examined by different incubation times. According to the research conducted by Prest et. al. (2013) where an incubation time of 5, 10 and 15 minutes at 35 °C for intact cell count with SGPI was used and due to the research done by Hammes et. al. (2012) that employed an incubation time of 5 minutes at 40 °C, it was decided to set the incubation time at 5 and 15 minutes for checking the amount of detected cells and stability of received results. Measurements were done every hour. Water sample used:

- Tap water (Sample 8). Results are shown in Figure 28.

Figure 28 shows that incubation time plays a prominent role in the accuracy of results. The amount of detected cells by 15 minutes incubation time was more stable in comparison to 5 minutes. The number of detected cells that where incubated for 5 minutes were 10 – 25 % more than the sample that where incubated for 15 minutes. The standard deviation for 15 minutes was 1700 count/ml (less than 5%) and 4200 count/ml (less than 10%) for 5 minutes of incubation. High nucleic acid cells (HNA) by both incubation times were in the same range.

After these results, the decision was to set the incubation time at 10 minutes and check another water sample in:

- Stand trial experiment in test mode. Results are shown in chapter 5.1.5 Test mode stand trial experiment;
- Real-time experiment in test mode. Results are shown in chapter 5.1.6 Test mode real-time experiment.

Results that are presented in chapters 5.1.5 and 5.1.6 showed that the incubation time at 10 minutes was good for next analyses in the field research (results are shown in chapter 5.2 Field research results) and for getting better separation of background.

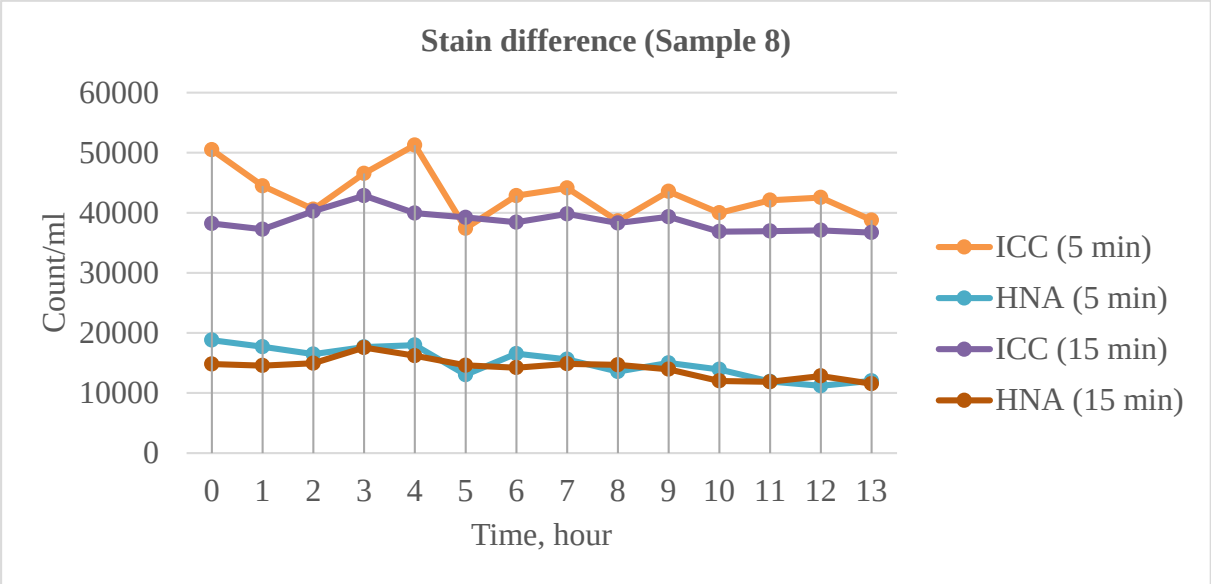

Figure 28: Stain difference Sample 8 results

5.1.2 Stability of stains

Following analysis aimed to check the stability of SG and SGPI stains. Newly prepared stains on 21.08.2017 and old stains made on 09.06.2017 were used. SGPI stain was prepared according to Table 1, and SG made according to Table 2. The time difference was two months. Measurements were performed with BD Accuri™ C6 Plus. Results for this analysis are shown in Figure 29 and Figure 30.

Results presented in Figure 29 and Figure 30 showed the excellent stability of old stain in comparison to the newly prepared. The difference in TTC for OBA old and freshly prepared SG stain was less than 2%. The difference in intact cell counts (SGPI stain) was less than 2%. The time difference between stains was two months, but storage of stain at 15 °C preserved the endurance of stains. The measurements showed that both stains estimated the same amount of bacterial cells.

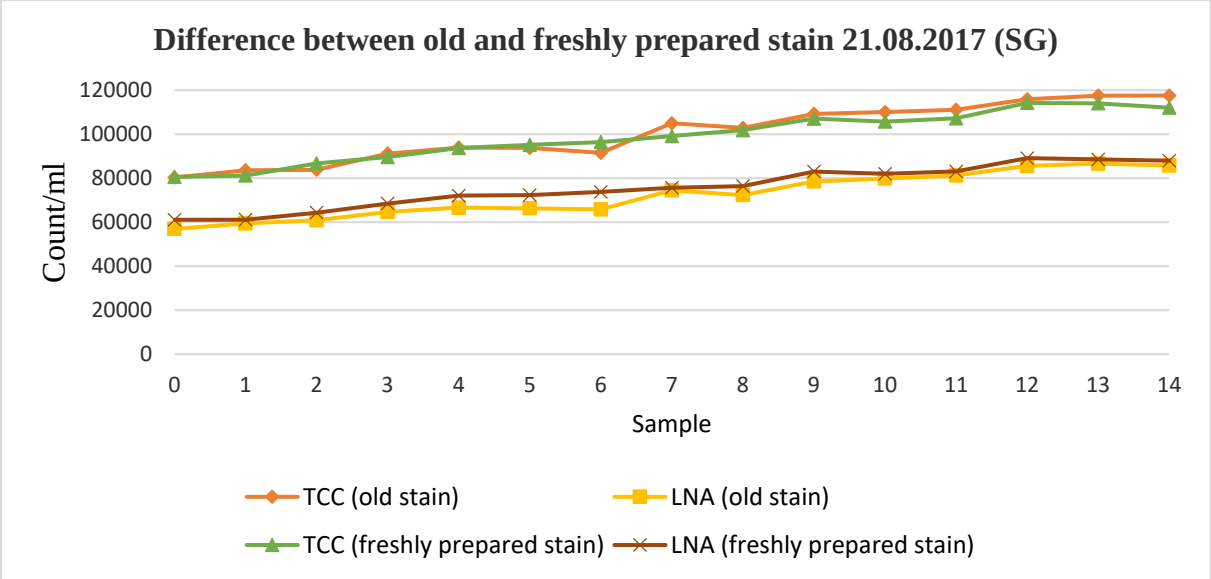

Figure 29: Difference between old and newly prepared stain 21.08.2017 (SG)

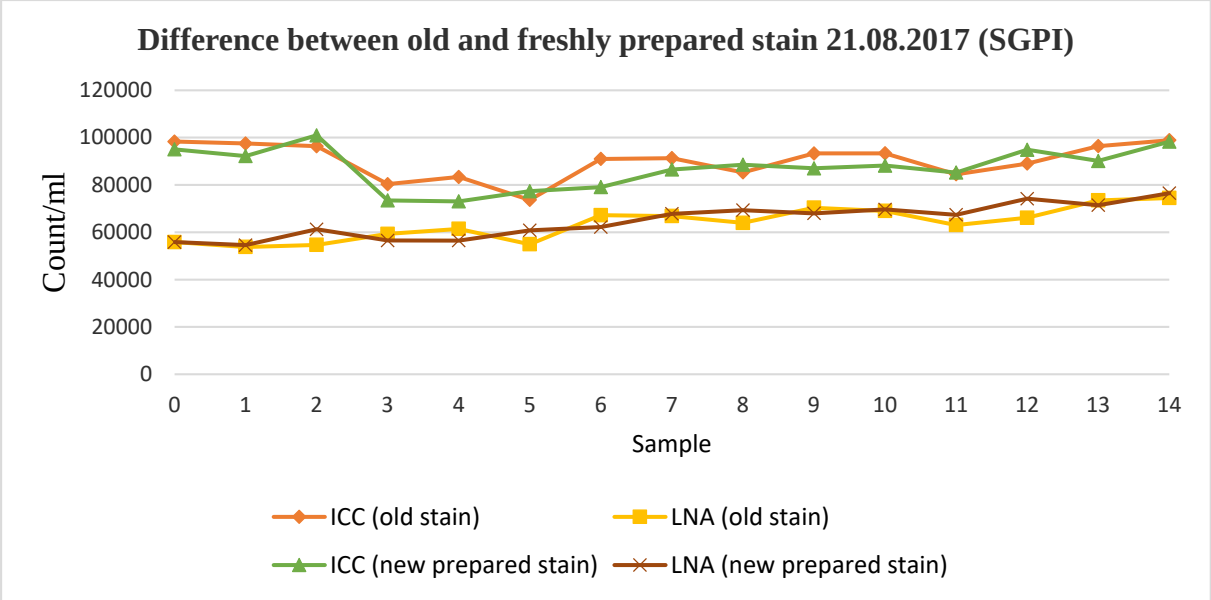

Figure 30: Difference between old and newly prepared stain 21.08.2017 (SGPI)

The analysis of stain stability was continued. Following measurements were done with old stains prepared on 09.06.2017 and newly prepared stains on 09.10.2017. SGPI stain was made according to Table 1, and SG made according to Table 2. The time difference between old and newly provided stains was four months. Results are shown in Figure 31 and Figure 32.

In Figure 31 and Figure 32 it can be seen that newly prepared SG and SGPI stain estimated a greater amount of cells in comparison to the old prepared stains. The difference in TTC for OBA old and freshly prepared SG stain was 2700 counts/ml (less than 4%). The difference in intact cell counts (SGPI stain) was 1000 counts/ml (less than 3%). This experiment showed that after four months of storage at 15 °C the stains estimated the number of cells close to the expected results.

Eventually it was decided to use stains no older than two months for obtaining representative data information about the real amount of cells in water samples. This decision was based on results presented in Figure 29 and Figure 30.

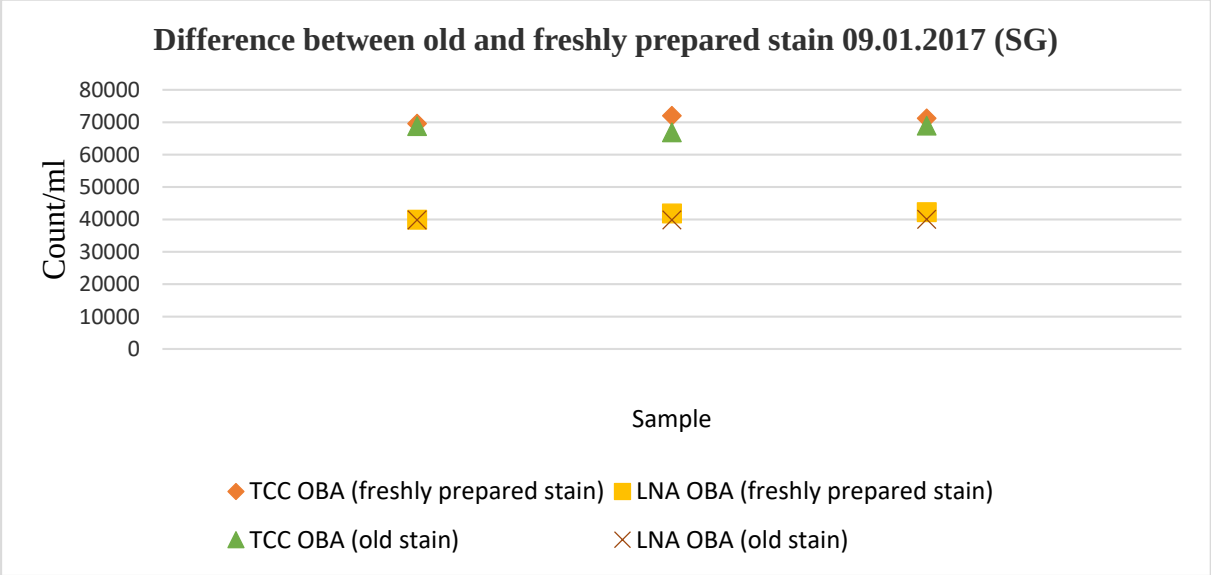

Figure 31: Difference between old and newly prepared stain 09.10.2017 (SG)

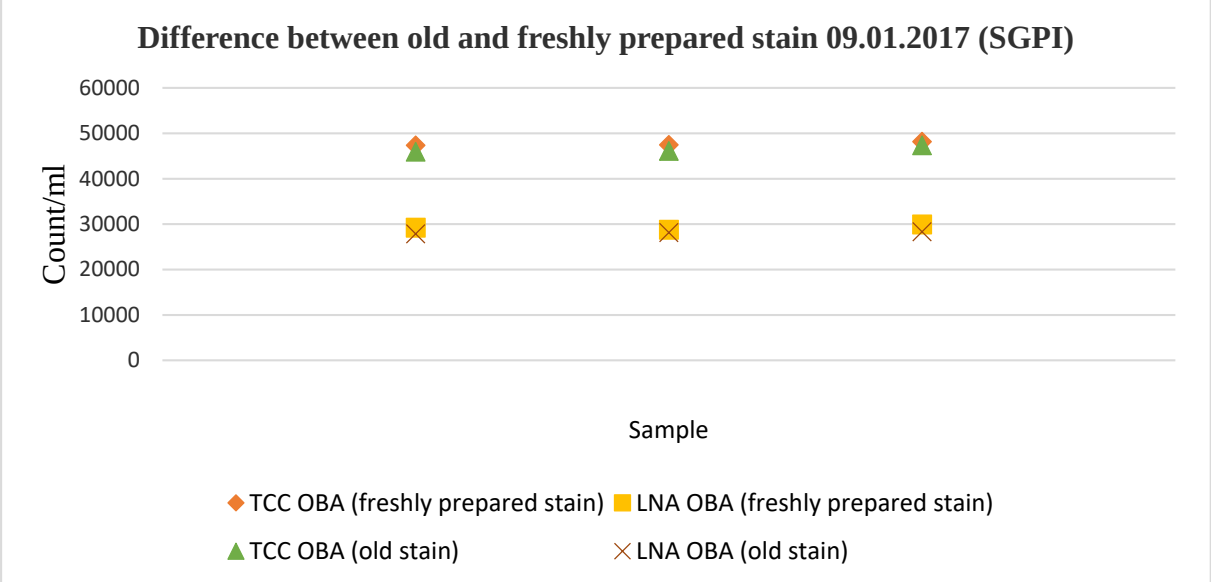

Figure 32: Difference between old and newly prepared stain 09.10.2017 (SGPI)

5.1.3 Calibration measurements

The aim of the conducted calibration measurements was to check OBA flow-cytometer on the stability of measuring. The measurements were performed with one peak calibration beads, due to the ability to form one cluster with a stable number of cells. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus and CyFlow® Cube 6. Results of reference measurements with all applied flow cytometers are listed in Table 22 below.

From the results of calibration measurements presented in Table 22 it can be seen that OBA measured the same amount of counts/ml as CyFlow® Cube 6. BD Accuri™ C6 Plus estimated +20% more counts/ml in comparison to all applied flow cytometers. The reason for the overestimated results by BD Accuri™ C6 Plus (+20% more counts/ml) was due to the individual feature of this equipment, in particular, the amount of sample volume for one analysis that the flow-cytometer was taken. The standard deviation by OBA was 1277 counts/ml and 1466 counts/ml by BD Accuri™ C6 Plus, which means that the obtained data values were approximately the same in the amount of variation. Results shown in Table 22 demonstrated that CyFlow® Cube 6 has had a lower standard deviation (514 counts/ml) in comparison to all applied flow-cytometers. Calibration beads measurements showed that OBA has had similar stability of measuring and this flow-cytometer could be applied in next experiments.

Table 22: Calibration beads measurements results

Number of measurement	OBA (counts/ml)	BD Accuri™ C6 Plus		CyFlow® Cube 6	
		(counts/ml)	Deviation to OBA result (%)	(counts/ml)	Deviation to OBA result (%)
1	28239	27185	-3.73	25820	-8.57
2	25959	29196	+12.47	24940	-3.93
3	25893	30877	+19.25	25870	-0.09
4	24772	30563	+23.38	24720	-0.21
5	25746	29056	+12.86	25400	-1.34
Average	26121	29375	-	25350	-
Standard deviation	1277	1466	-	514	-

5.1.4 Effect of system cleaning

The cleaning of the system was an essential tool after making a set of measurements in the research; therefore, the cleaning of the system was a point of discussion because of the OBA, by sample port cleaning the pure water sprayed in to the sample. The pure water was sprayed in to the sample port either by “pure water” or by “cleaning solution” application. This analysis is aimed to check the influence of this cleaning process on the obtained results.

The OBA flow-cytometer was automatically cleaned in the field research after the defined amount of measurements. It was necessary to check the influence of system cleaning on further results, which are shown in the chapter: 5.2 Field research results.

5.1.4.1 Effect of system cleaning with pure water

For checking the possibility of influence of the cleaning process with the pure water that was introduced on the sample, measurements were performed. The following analysis was carried out every hour. The cleaning was done after 5 hours of measurements. The incubation time was set at 5 min for Stain B (according to the results shown in chapter: 5.1.1.1 Stain and incubation times for TCC determination).

Water samples used: UV-C processed water. Results are shown in the Table 23. These results are also shown in the Figure 33.

Results shown in Table 23 and Figure 33 demonstrated that TCC in UV-C processed water sample at the beginning of the experiment (first 5 hours) was in the same range with the average of 127000 counts/ml. After 5 hours of experiment, the cleaning application with pure water was performed. The amount of detected cells after cleaning was slightly decreased from 125844 counts/ml (before cleaning starts) to 123755 counts/ml (after cleaning with pure water). The second result after cleaning also demonstrated less obtained cells (122192 counts/ml). This changes could be recognized in the Figure 33. The third measurement after cleaning with pure water was similar to the values that were obtained before cleaning application. After the third measurement, the results demonstrated a linear trend.

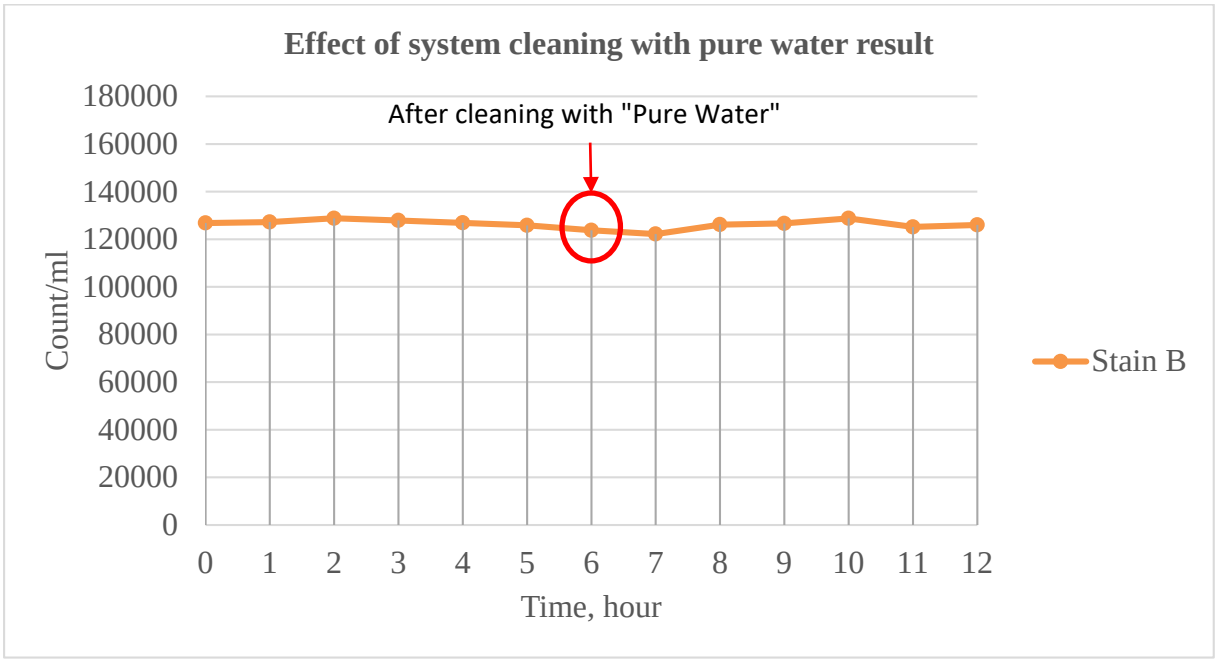

Figure 33: Effect of system cleaning with pure water result

Table 23: Effect of system cleaning with pure water result

Time, hour	Stain B (count/ml)
0	126822
1	127222
2	128814
3	127896
4	126881
5	125844
Pure Water cleaning	
6	123755
7	122192
8	126170
9	126666
10	128792
11	125192
12	126037

This experiment showed, that cleaning with pure water slightly reduced the amount of detected cells promptly after cleaning. The measurement confirmed that there was influence by cleaning with pure water. The reason for these changes was due to the residuals of pure water which remained in the system after cleaning. It means that after cleaning the pure water dilutes with the next samples and influence the final result.

Due to the obtained results it was decided to check the influence of cleaning with cleaning solution (results are shown in the chapter: 5.1.4.2 Effect of system cleaning with cleaning solution). The results by applying cleaning with and without pure water are shown in the chapter: 5.2.1 Research mode stand trial experiment.

5.1.4.2 Effect of system cleaning with cleaning solution

After conducted analysis of system cleaning (shown in the chapter: 5.1.4.1 Effect of system cleaning with pure water) it was decided to perform next analysis of the system cleaning with the cleaning solution. This analysis was aimed to check the influence of cleaning solution application on the obtained results.

The following analysis was carried out every hour. The cleaning was done after 10 hours of measurements. The incubation time was set at 5 min for Stain B (according to the results shown in chapter: 5.1.1.1 Stain and incubation times for TCC determination). The water sample that was used: untreated raw water. Results are shown in Figure 34.

Results shown in Figure 34 demonstrated that TCC in the water sample at the beginning of the experiment (first 10 hours) was in the same range with an average 37000 counts/ml. After 10 hours of experiment, the cleaning application with cleaning solution was conducted. The amount of detected cells after cleaning was strongly decreased from 37192 counts/ml (before cleaning starts) to 26696 counts/ml (after cleaning application). The second and the third result after cleaning also demonstrated less obtained cells (32429 and 32377 counts/ml respectively). This changes could be recognized in the Figure 34. The fourth measurement after cleaning with cleaning solution was similar to the values that were obtained before cleaning application. After the fourth measurement, the results were returned to the same values, which were before cleaning application started.

This experiment showed, that cleaning with cleaning solution reduces the amount of detected cells promptly after cleaning. The measurement confirmed that there was influence by cleaning with cleaning solution. The reason for these changes was due to the residuals of cleaning solution and the pure water which remained in the system after cleaning. Due to the obtained results, it was decided to perform cleaning with cleaning solution only for measurements in the flow (at the waterwork). The results by applying cleaning with the cleaning solution are shown in the chapter: 5.2.2 Research mode real-time experiments.

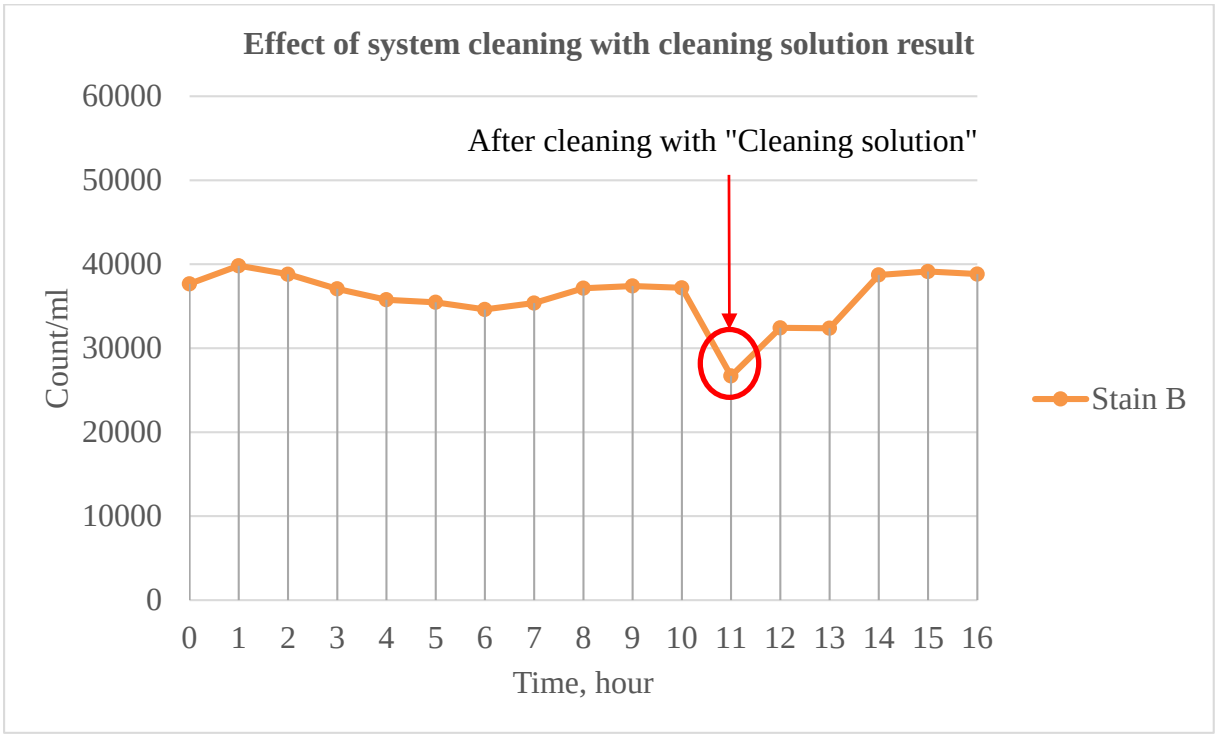

Figure 34: Effect of system cleaning with cleaning solution result

5.1.5 Test mode stand trial experiment

The aim of the stand trial experiment was to analyse the growth of bacteria in raw water.

For analysis two differently prepared stains were used:

- Stain A – SYBR Green propidium iodide prepared at BOKU (for preparation steps and composition see Table 1;
- Stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2).

Incubation times were:

- Stain A by 10 min. The incubation time was set according to the obtained results that are shown in chapter: 5.1.1.2 Stain and incubation times for ICC determination;
- Stain B by 5 min. According to the obtained results that are shown in chapter: 5.1.1.1 Stain and incubation times for TCC determination.

Measurements were done every hour. Water samples used:

- Well water. Results are listed in Figure 35.

Results show that the changes in the number of cells could be rapidly recognised. An increase in ICC for stain A from 320000 to 420000 counts/ml and in TCC for stain B from 456000 to 610000 counts/ml was noticed. A slight growth in HNA for both stains was also observed. HNA changed for stain A from 90000 to 157000 counts/ml and for stain B from 73000 to 147000 counts/ml. The reason for these changes was due to the not conducted cleaning application with pure water. After this analysis, it was decided to perform cleaning application with pure water for water samples with high number of cells. The results by applying cleaning with and without pure water are shown in the chapter: 5.2.1 Research mode stand trial experiment. Results shown in Figure 35 demonstrated that the incubation time for both stains was appropriate for water sample analysis. According to the obtained results it was decided to perform next analysis with defined incubation time in the research mode (results are shown in chapter: 5.2.1 Research mode stand trial experiment).

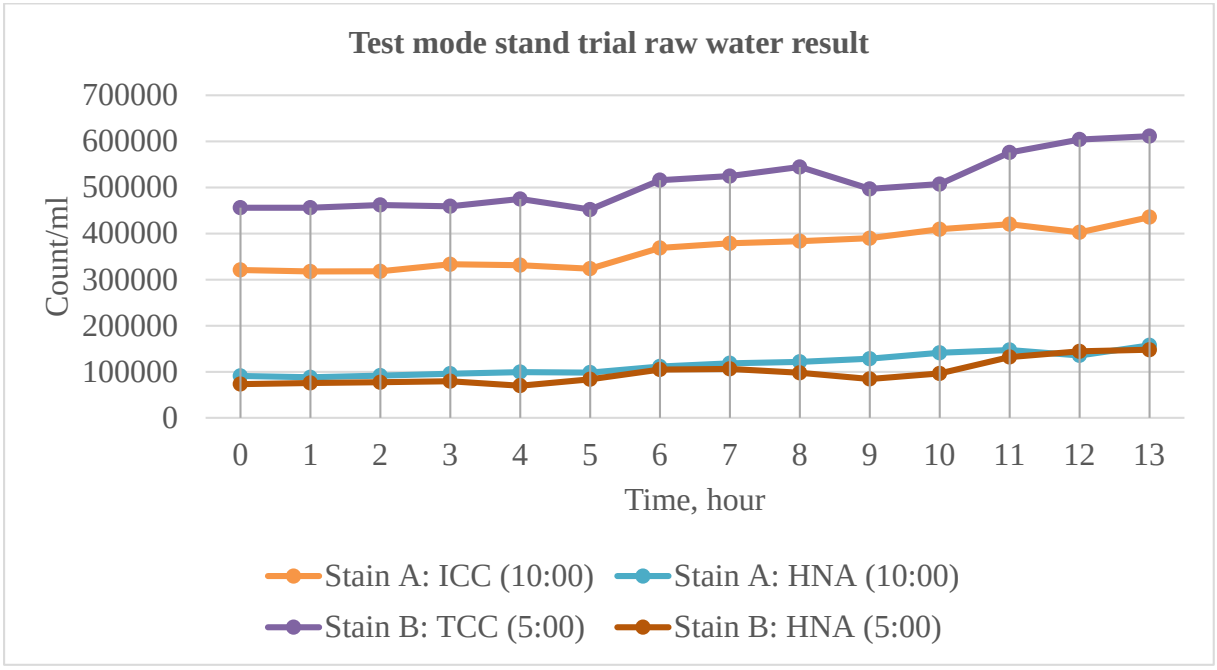

Figure 35: Test mode stand trial raw water results

5.1.6 Test mode real-time experiment

The aim of this analysis was to monitor microbiological changes in the tap water. Real-time results can be affected by many factors. Therefore, the flow of water was adjusted at 25 L/hour, for avoiding growth of cells in a hose. For the analysis the incubation time for stain A and stain B that was previously defined in chapter: 5.1.5 Test mode stand trial experiment was used.

Measurements were done every two hours. Water samples used:

- Tap water that was connected to the hose. Results are shown in Figure 36.

Results depicted in Figure 36 show a high number of cells at the beginning of the experiment because the water had been stagnating for too long in the distribution pipeline and there was a possibility of the growth of bacteria. After 4 hours the number of cells was coming to a more realistic number. After 24 hours of measurements, there were no visible changes in water quality. Only the number of detected cells for both stains has slightly increased. ICC changed for stain A from 15000 to 20000 counts/ml and in TCC for stain B from 35000 to 45000 counts/ml. After 26 hours of measurements it was observed that TCC by stain B continually changed. The reason for such fluctuation was that the flow speed of water was lower than 25 L/hour, and there was a possibility of air reaching into the system. For the next analysis it was decided to set the flow speed at 25 L/hour.

After the test mode in the laboratory it was decided to check water quality in the flow every 20 minutes at waterwork (results are shown in chapter: 5.2.2 Research mode real-time experiments).

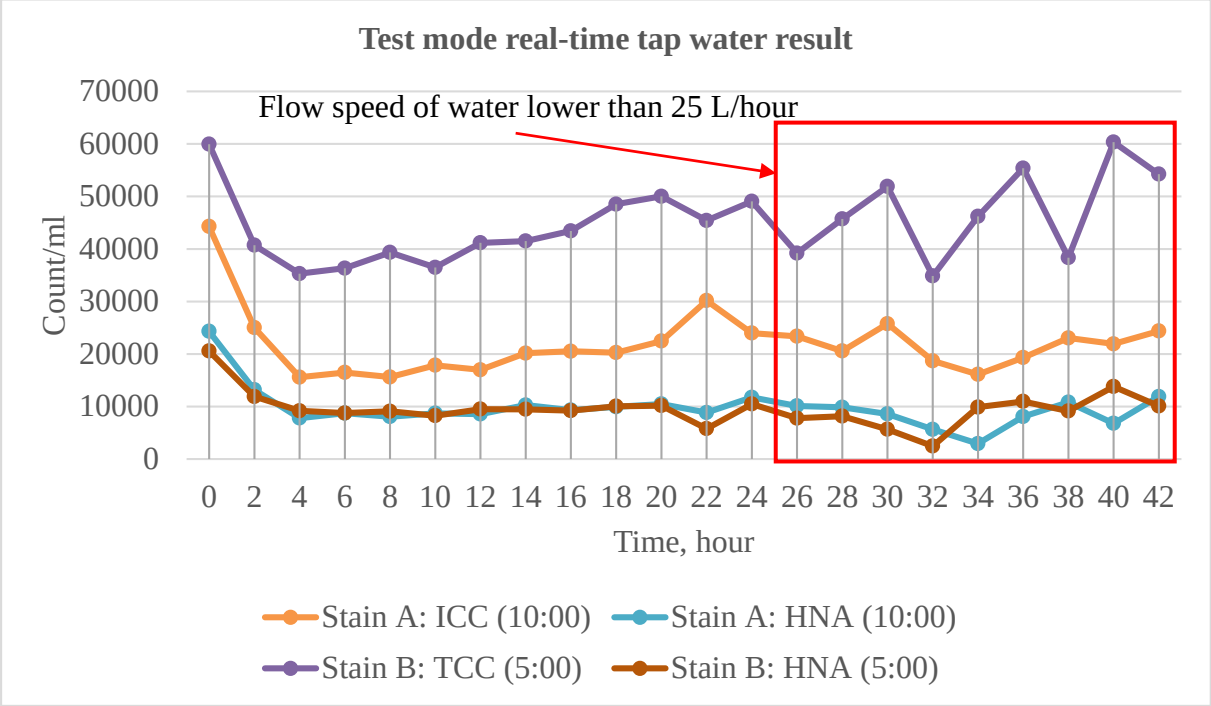

Figure 36: Test mode real-time tap water results

5.2 Field research results

5.2.1 Research mode stand trial experiment

The aim of the stand trial experiment in the research mode was to analyse the growth of bacteria in UV-C processed water. The duration of each stand trial experiment was seven days. This time interval was chosen for exploring growth of cells in UV-C processed water, because in normal cases the distribution of water to the consumer is carried out during this period of time. Measurements were done every hour. Measurements were started when water sample reached 15 °C. Before the experiment started, the system was cleaned with cleaning solution. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus.

For analysis two differently prepared stains were used:

- Stain A – SYBR Green propidium iodide prepared at BOKU (for preparation steps and composition see Table 1;
- Stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2).

Incubation times were:

- Stain A by 10 min. The incubation time was set according to the obtained results that are shown in chapter: 5.1.1.2 Stain and incubation times for ICC determination5.1.1.2 Stain and incubation times ICC;
- Stain B by 5 min. According to the obtained results that are shown in chapter: 5.1.1.1 Stain and incubation times for TCC determination.

5.2.1.1 Stand trial experiment without cleaning

The aim of the conducted stand trial experiments without cleaning was to check results which were obtained in the chapter: 5.1.5 Test mode stand trial experiment. Moreover, it was decided to continue analysis of the system cleaning which was previously observed in the chapter: 5.1.4.1 Effect of system cleaning with pure water.

Results of one week of regular measurements with SG out of a 2 L bottle with UV-C processed water, which was placed in the incubator at 15 °C, are shown in Figure 37. At the same time, this water was also measured with SGPI stain; results are shown in Figure 38.

Results in Figure 37 show an increase of TCC from 48000 to 100000 counts/ml. For the first three days, there was no growth which means that TCC, LNA and HNA were on the same level. The average for TCC, for the first three days, was 53300 counts/ml and standard deviation 4300 counts/ml (less than 5%). On the third day the slight growth of counts/ml by TCC and HNA was seen. LNA stayed at the same level with an average of 35000 counts/ml. On the third day of measurements HNA and LNA counts/ml were on the same level, and afterwards, HNA was slightly rising till the end of the experiment. Over the period, HNA changed from 20000 to 59000 counts/ml.

Figure 38 shows the same tendency as Figure 37. The difference is only in the counts/ml. By ICC the number of detected cells varied from 32000 to 55000 counts/ml. The average for ICC, for the first three days, was 35000 counts/ml and standard deviation 2600 counts/ml (less than 5%). HNA was slightly rising from 12000 to 33000 counts/ml. LNA was constant during the whole period of measurements; the average value was 21000 counts/ml.

Afterwards, the system was cleaned with cleaning solution and the experiment was repeated with another UV-C processed water sample that was received from the waterwork. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus. The results are shown in Figure 39 and Figure 40.

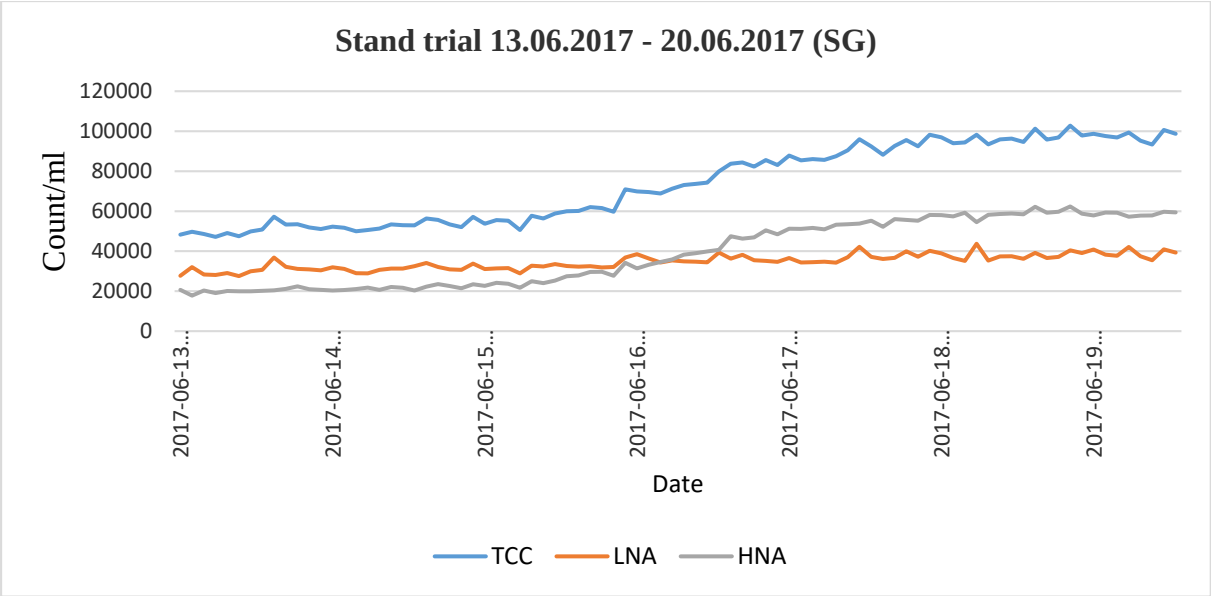

Figure 37: Research mode stand trial experiment 13.06.2017 - 20.06.2017 (SG)

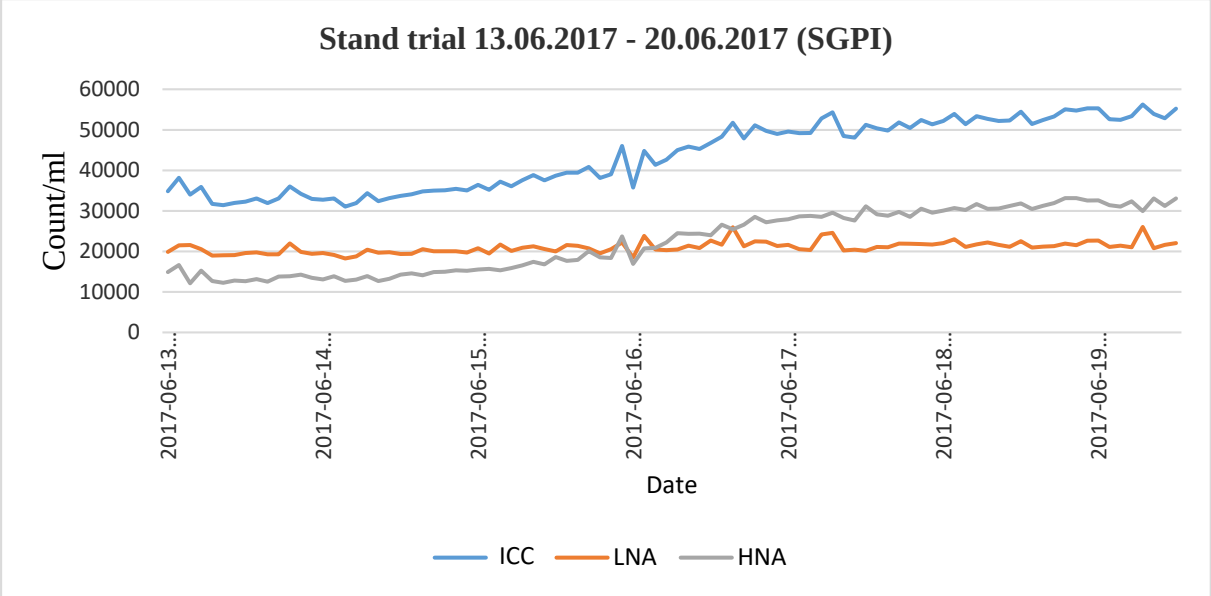

Figure 38: Research mode stand trial experiment 13.06.2017 - 20.06.2017 (SGPI)

After obtained results of one week of regular measurements with SG and SGPI were obtained, which are shown in Figure 37 and Figure 38, it was decided to repeat this analysis with new UV-C processed water sample received from the waterwork and to perform reference measurements with the flow cytometer BD Accuri™ C6 Plus. Reference measurements were essential for checking the effect of not performed cleaning of the system. The results are shown in Figure 39 and Figure 40.

Figure 39 shows the same tendency for the growth of cells like Figure 37. The difference was only in values. TCC slightly increased from 29000 to 118000 counts/ml. For HNA initial measurements stood at 11000 counts/ml whereas final measurements indicated the value of 80000 counts/ml. As in research done between 13.06.2017 – 20.06.2017 (Figure 37) the same growth of cells after the third day of measurements was observed.

Figure 40 display similar tendency for the experiment with SGPI stain. The values for ICC ranged from 20000 to 60000 counts/ml. HNA slightly changed from 8000 to 40000 counts/ml. LNA showed no changes in value with an average of 15000 counts/ml.

The reference measurements with BD Accuri™ C6 Plus (shown in Figure 39) were conducted on the day 0, day 3 and day 7. TCC on the day 0 was 29000 counts/ml, which was similar to OBA results. On the day the TCC by BD Accuri™ C6 Plus was 29800 counts/ml and by OBA was 48300 counts/ml (60% deviation). At the end of this experiment (day 7) the TCC by reference measurement was 48000 counts/ml and by OBA was 118000 counts/ml. The number of detected cells by OBA was higher in comparison to reference measurements with BD Accuri™ C6 Plus. This difference in the results was either due to contamination of water sample or because of not performed cleaning of the system. After this experiment, it was decided to repeat the stand trial experiment with reference measurements for finding out the reason for the difference in the obtained results.

Afterwards, the system was cleaned with cleaning solution, and another UV-C processed water sample, that was received from the waterwork, was measured. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus. The results are shown in Figure 41 and Figure 42.

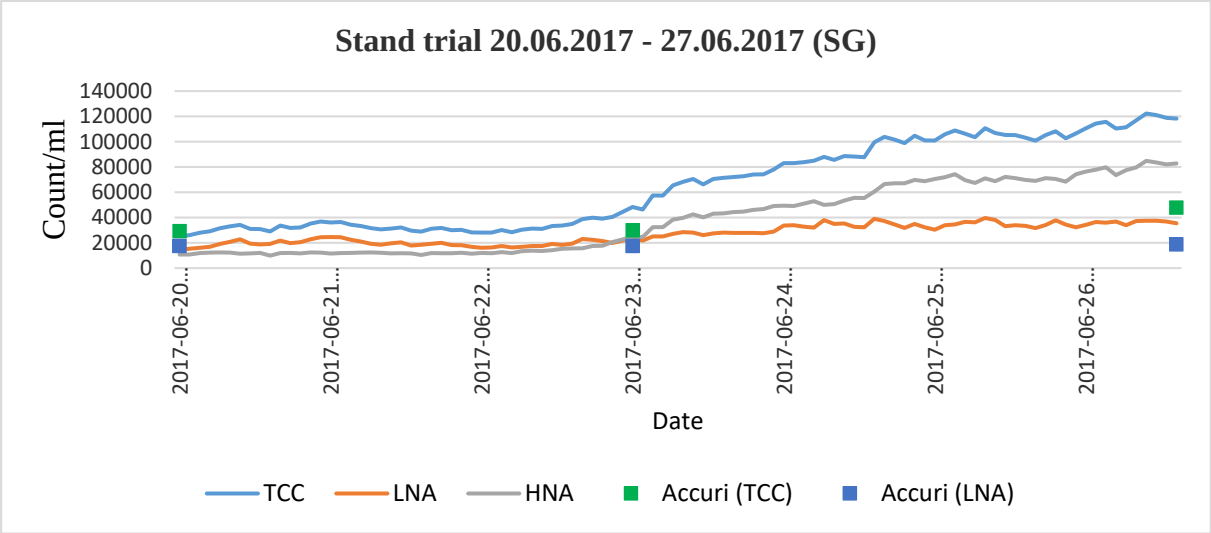

Figure 39: Research mode stand trial experiment 20.06.2017 - 27.06.2017 (SG)

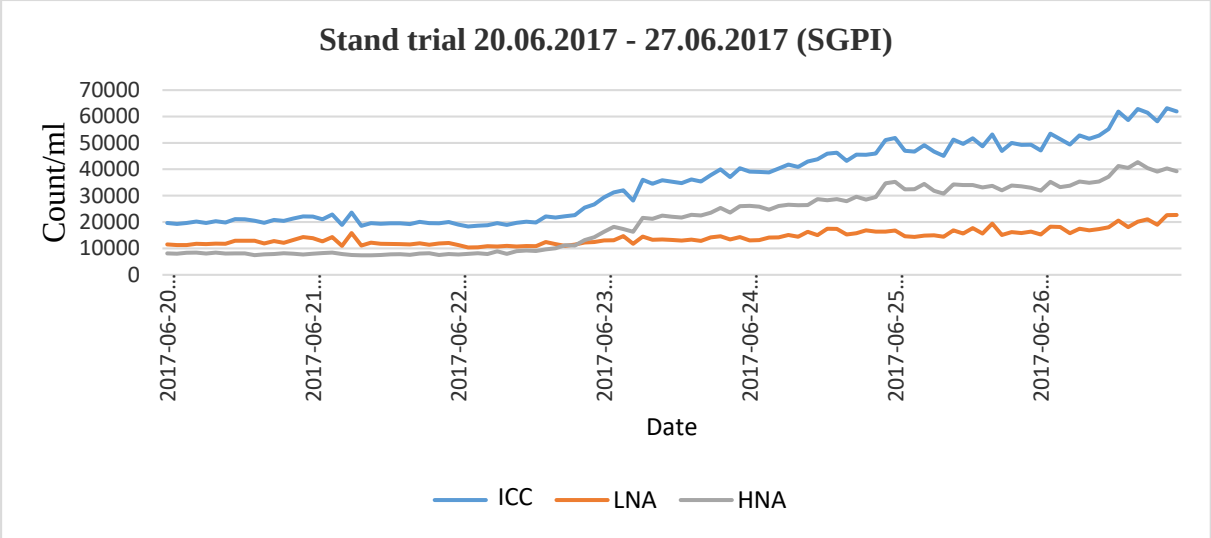

Figure 40: Research mode stand trial experiment 20.06.2017 - 27.06.2017 (SGPI)

After obtained results of previous week of regular measurements with SG and SGPI shown in Figure 39 and Figure 40 it was decided to repeat this analysis with new UV-C processed water sample received from the waterwork. This experiment was aimed to find out the reason for the difference in results between OBA and reference measurements with BD Accuri™ C6 Plus, which are shown in Figure 39. The results are shown in Figure 41 and Figure 42.

Figure 41 shows visible growth of TCC from 35000 to 60000 counts/ml. As well as in previous experiments growth of cells started on the third day. During the first three days of the experiment, the results were stable and no changes in growth were observed. The average for TCC, for the first three days, was 40000 counts/ml and standard deviation 3700 counts/ml (less than 5%). Afterwards a slight growth in TCC, LNA and HNA was observed. LNA changed from 20000 to 33000 counts/ml. HNA showed changes in growth after the fourth day of the experiment with the value rising from 18000 to 27000 counts/ml.

Figure 42 displays the growth of intact cells. The first three days of the analysis showed changes in ICC, LNA and HNA. After the third day ICC changed from 25000 to 43000 counts/ml. LNA shows a very slight growth of cells from 13000 to 20000 counts/ml. The first four days of the experiment signalled no significant changes in HNA. Afterwards, HNA rose and intersected with LNA trendline. On the fifth day, HNA was higher than LNA, and this tendency continued till the end of the experiment.

The reference measurements with BD Accuri™ C6 Plus (shown in Figure 41) were conducted on the day 0 and day 7. TCC on the day 0 was similar to OBA results (35000 counts/ml). On the day 7 the TCC by reference measurement was 30700 counts/ml and by OBA was 62600 counts/ml. The number of detected cells by OBA was twice as high in comparison to reference measurements with BD Accuri™ C6 Plus. This difference in the results was due to not performed cleaning of the system. After this experiment, it was decided to repeat the stand trial experiment with reference measurements for confirming the reason for the difference in the obtained results shown in Figure 41.

Afterwards, the system was cleaned with cleaning solution, and another UV-C processed water sample, that was received from the waterwork, was measured. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus. The results are shown in Figure 43 and Figure 44.

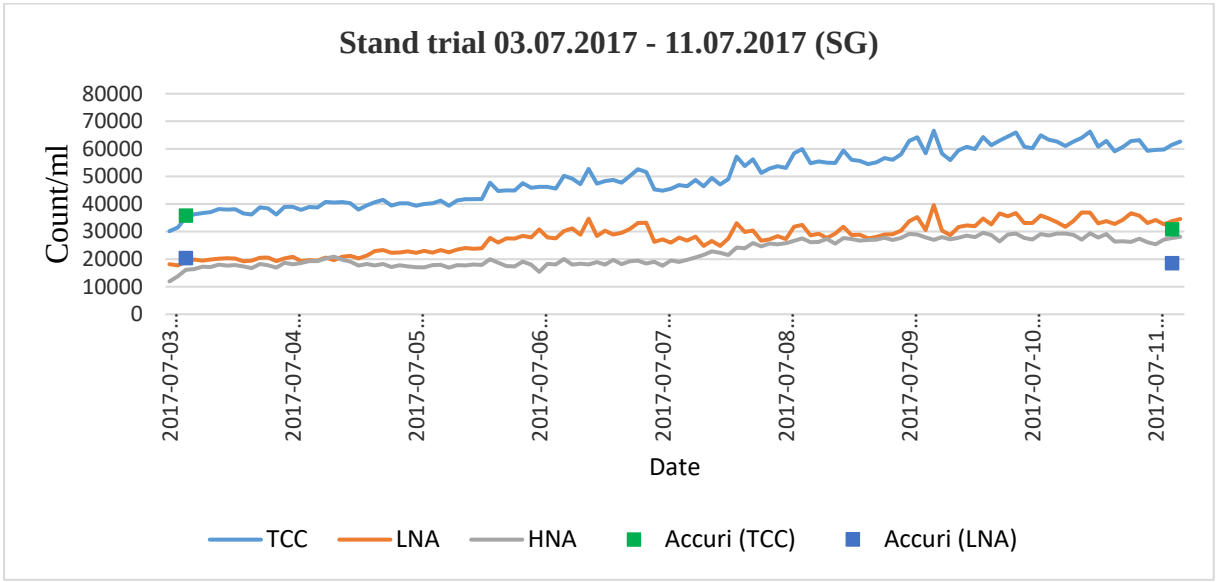

Figure 41: Research mode stand trial experiment 03.07.2017 - 11.07.2017 (SG)

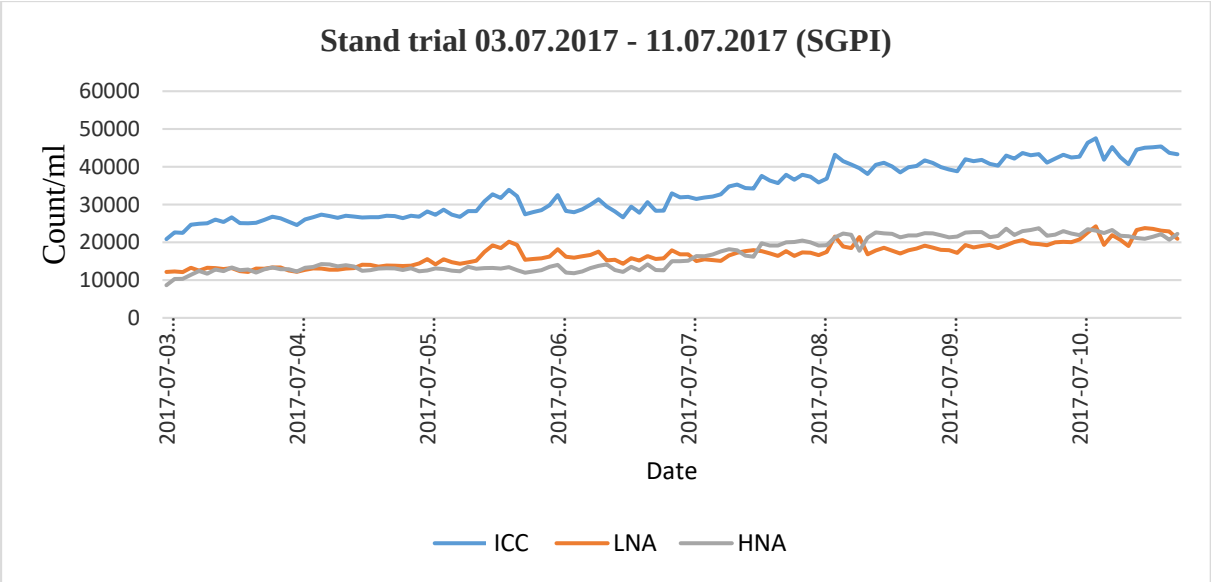

Figure 42: Research mode stand trial experiment 03.07.2017 - 11.07.2017 (SGPI)

After obtained results of previous week of regular measurements with SG and SGPI shown in Figure 41 and Figure 42 it was decided to repeat this analysis with new UV-C processed water sample received from the waterwork. This experiment was aimed to confirm the reason for the difference in results between OBA and reference measurements with BD Accuri™ C6 Plus, which are shown in Figure 41. The results are shown in Figure 43 and Figure 44.

Figure 43 shows no changes during the first three days of the experiment. The average for TCC, for the first three days, was 39600 counts/ml and standard deviation 3300 counts/ml (less than 5%). Later TCC grew from 35000 to 60000 counts/ml. Number for LNA ranged between 18000 to 35000 counts/ml. HNA was somewhat stable and only on the last day of the experiment a slight rise from 20000 to 24000 counts/ml was detected.

Figure 44 shows no observed changes during the first three days of the experiment. The average for ICC, for the first three days, was 26900 counts/ml. On the third day the growth of cells in ICC increased from 23000 to 40000 counts/ml. LNA and HNA were on the same level during the whole experiment and had very slight rise from 12000 to 20000 counts/ml.

The reference measurements with BD Accuri™ C6 Plus (shown in Figure 43) were performed on the day 0 and day 7. TCC on the day 0 was similar to OBA results (28000 counts/ml). On the day 7 the TCC by reference measurement was 25000 counts/ml and by OBA was 62000 counts/ml. The number of detected cells by OBA was twice as high in comparison to reference measurements with BD Accuri™ C6 Plus (similar to obtained results shown in Figure 41). This difference in the results was for sure due to not performed cleaning of the system.

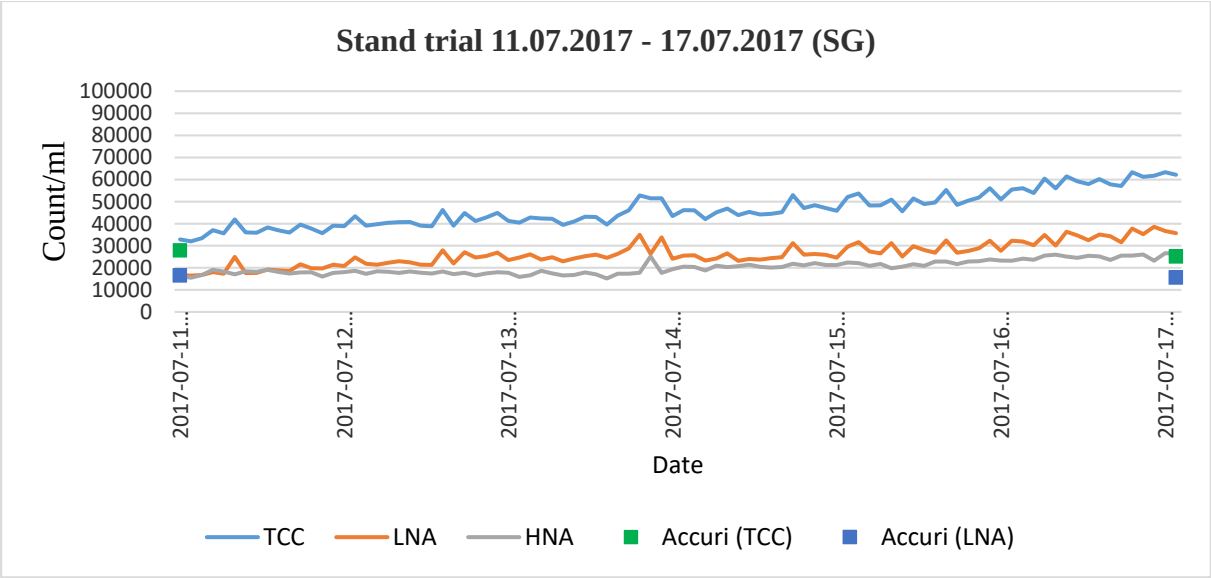

Figure 43: Research mode stand trial experiment 11.07.2017 - 17.07.2017 (SG)

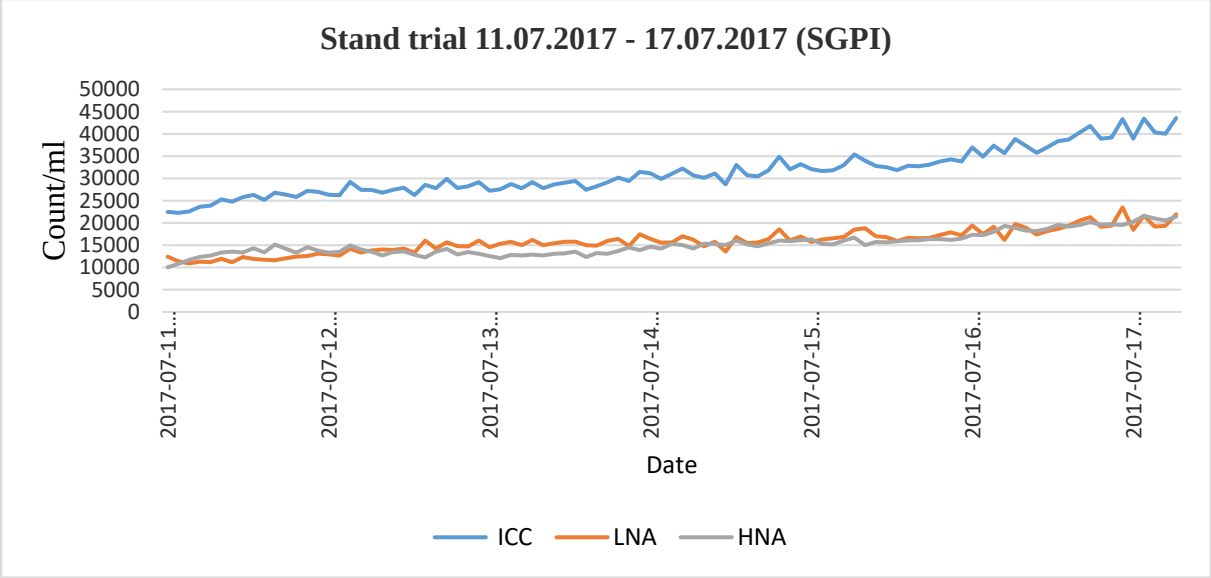

Figure 44: Research mode stand trial experiment 11.07.2017 - 17.07.2017 (SGPI)

The analyses showed the growth of bacteria from measurements performed with OBA (Figure 37 – Figure 44) but did not demonstrate growth in reference measurements with BD Accuri™ C6 Plus. The increase in the number of detected cells by OBA was due to the not performed cleaning of the system during the experiment. After conducted analyses without cleaning it was decided to perform stand trial experiment with cleaning of the system every 24 hours with pure water and to take reference measurements on a daily basis with the same water sample with BD Accuri™ C6 Plus. The results are shown in chapter: 5.2.1.2 Stand trial experiment with pure water cleaning.

5.2.1.2 Stand trial experiment with pure water cleaning

Next stand trial experiment was done with UV-C treated water. Cleaning of the system was done every 24 hours with pure water. This experiment aimed to take reference measurements with the same water sample with BD Accuri™ C6 Plus. Data of stand trial measured with OBA is represented in Figure 45. The results of the reference measurements with the same water sample are shown in Figure 46.

Results shown in Figure 45 demonstrated no changes in the number of cells for the three first days of the experiment. After the third day a slight growth of bacteria in TCC from 125000 to 166000 counts/ml was noticed. The LNA cells did not change during the whole trial. Slight growth of HNA cells was indicated after the third day of the experiment. The growth of bacterial cells was not high in comparison to received results shown in Figure 37 – Figure 43. The reason for these differences in the number of bacterial cells was due to the not conducted cleaning application with pure water. After this analysis, it was decided to perform cleaning application with pure water for stand trial experiments every 24 hours (results are shown in Figure 47).

Reference measurements with BD Accuri™ C6 Plus represented in Figure 46 showed no changes in the number of cells. The TCC during the whole experiment has slightly fluctuated, but overall there was no change in the number of cells. The TCC fluctuated from 165000 to 200000 counts/ml. BD Accuri™ C6 Plus estimated +20% more counts/ml in comparison to OBA results shown in Figure 45. The reason for the overestimated results by BD Accuri™ C6 Plus (+20% more counts/ml) was due to the individual feature of this equipment (results of calibration measurements are shown in the chapter: 5.1.3 Calibration measurements). According to the obtained results, it was decided to perform next stand trial analysis for 14 days with water treated with 0.1 mg/L ClO_2.

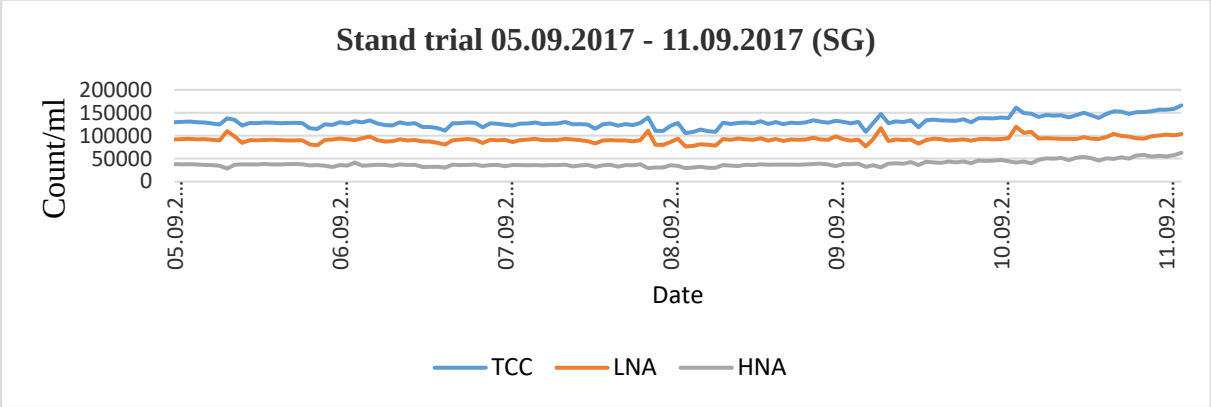

Figure 45: Research mode stand trial experiment 05.09.2017 – 11.09.2017 (SG)

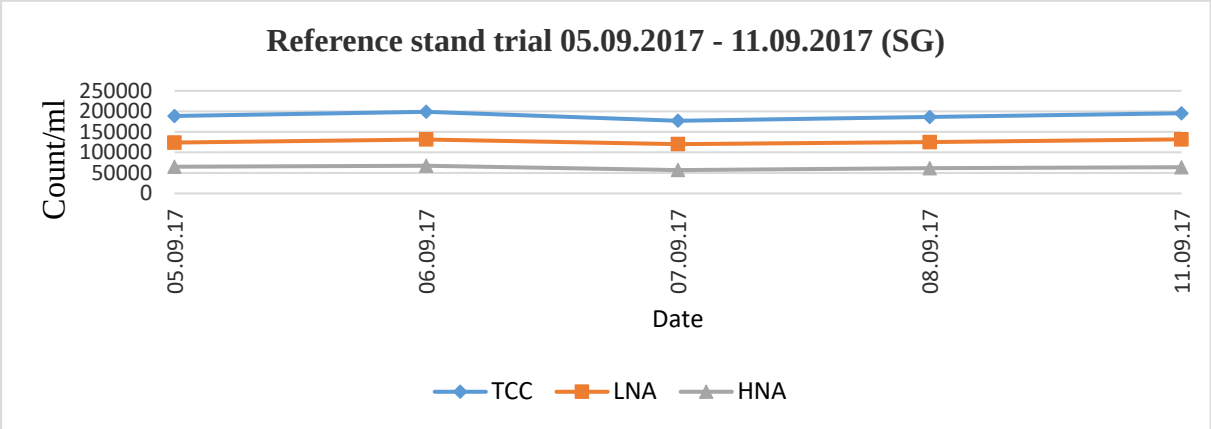

Figure 46: Reference for stand trial experiment 05.09.2017 – 11.09.2017 (SG)

The final stand trial experiment was done for 14 days with processed water with 0.1 mg/L chlorine dioxide. Cleaning of the system was done every 24 hours with pure water. The results are represented in Figure 47.

Figure 47 shows the growth of bacteria during 14 days. In the first seven days of the experiment no considerable changes in the growth of bacteria were noticed. The average for TCC, for the first seven days, was 29000 counts/ml and standard deviation 5300 counts/ml (less than 5%). After day seven until day ten slight growth of bacteria was indicated. The TCC in this period changed from 30000 to 65000 counts/ml. After day ten until day 12 an extreme increase of TCC from 65000 to 650000 counts/ml was detected.

The reference measurements with BD Accuri™ C6 Plus (shown in Figure 47) were performed on the day 0, 1, 2, 6, 7, 8, 9, 10, 13 and day 14. TCC on the day 0, 1, 2, 6, 7 was similar to OBA results (35000 counts/ml). BD Accuri™ C6 Plus estimated (on the day 0, 1, 2, 6, 7) +20% more counts/ml in comparison to OBA results due to the individual feature of this equipment (results of calibration measurements are shown in the chapter: 5.1.3 Calibration measurements). From day 0 to day 14 the number of detected cells from the reference measurements was in the same range (no increase in bacteria was observed), compared to the OBA results. The difference in the results was caused by contamination of the equipment, which was measured by OBA.

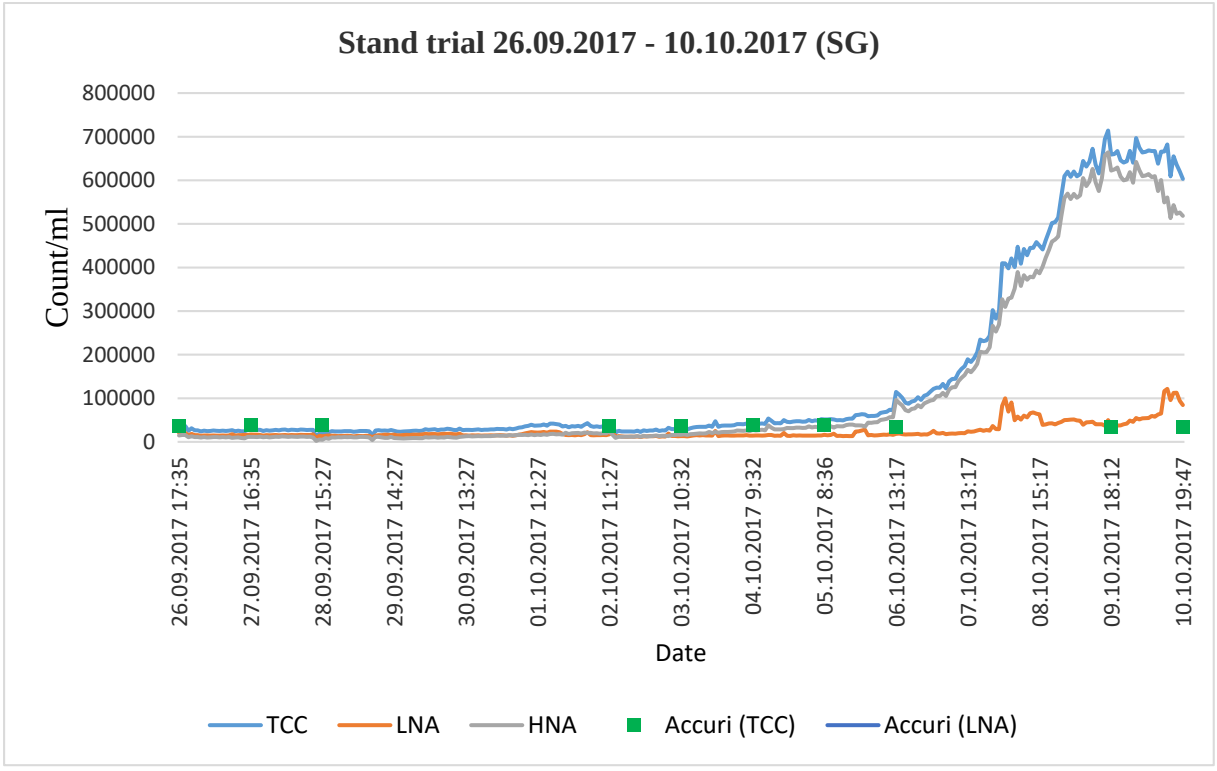

Figure 47: Research mode stand trial experiment 26.09.2017 – 10.10.2017 (SG)

Stand trial experiments with pure water cleaning showed good applicability of OBA. The growth of bacteria was measured every hour which made experimental data more relevant. The cleaning application with pure water which was performed every 24 hours in stand trial experiments (results are listed in Figure 45 and Figure 47) showed better results for measuring the growth of bacterial cells. According to the obtained results, it was decided to perform cleaning with pure water every 24 hours for stand trial experiments.

5.2.2 Research mode real-time experiments

The aim of the real-time experiments in the research mode (at the waterwork) was to monitor microbiological changes in the untreated raw water. Measurements were done with continuous sampling every 20 minutes. The duration of each experiment was seven days. This time interval was chosen for better presentation of the received results. Before the experiment started, the system was cleaned with cleaning solution. The flow speed of water was adjusted at 25 L/hour, for avoiding growth of cells in a hose (according to the obtained results that are shown in chapter: 5.1.6 Test mode real-time experiment). Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus and CyFlow® Cube 6.

For analysis two differently prepared stains were used:

- Stain A – SYBR Green propidium iodide prepared at BOKU (for preparation steps and composition see Table 1;
- Stain B – SYBR Green prepared at BOKU (for preparation procedure and composition see Table 2).

Incubation times were:

- Stain A by 10 min. The incubation time was set according to the obtained results that are shown in chapter: 5.1.1.2 Stain and incubation times for ICC determination;
- Stain B by 5 min. According to the obtained results that are shown in chapter: 5.1.1.1 Stain and incubation times for TCC determination.

5.2.2.1 Real-time experiments without cleaning

The aim of the conducted real-time experiment without cleaning over 7 days was to check results which were obtained in the chapter: 5.1.6 Test mode real-time experiment. Moreover, it was decided to continue analysis of the system cleaning which was previously observed in the chapter: 5.1.4.2 Effect of system cleaning with cleaning solution.

The results of one week of regular measurements with SG and SGPI of the untreated raw water (at the waterwork) are shown in Figure 48 and Figure 49. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus.

Figure 48 shows an increase of the number of cells at the beginning of the experiment, due to the fact that the system had previously been cleaned and there was a little amount of cleaning solution in the system, which resulted in an underestimation of the real amount of cells in a water sample (according to the obtained results shown in the chapter: 4.2.4.2 Effect of system cleaning with cleaning solution). Afterwards, results were stable during the whole experiment. TCC average was 40000 counts/ml and 23000 count/ml for LNA (the standard deviation was less than 5%). Several rises of cells on the third day of the experiment were observed. The reason for these changes was a variation of the flow speed for untreated raw water. Measurements with SGPI stain are shown in Figure 49.

Figure 49 shows a strong growth of cells at the beginning of the research. Afterwards stable results for measurements were observed. From the third day of the experiment, there were several fluctuations of ICC and LNA because measurements were done in flow and there was a possibility that the flow speed of untreated raw water varied. Nevertheless, ICC and HNA showed no significant difference. ICC average during the research was 30000 counts/ml and 20000 count/ml for LNA (the standard deviation was less than 5%).

The reference measurements with BD Accuri™ C6 Plus (shown in Figure 48) were conducted on the day 0, 1, 2, 3 and day 7. TCC on the day 0 and day 1 showed similar values of detected cells. The deviation between these obtained results (day 0 and day 1) was less than 5%. Starting from the second day, the number of detected cells by the reference measurement differed from

the result obtained by OBA. The number of detected cells by OBA was higher in comparison to reference measurements with BD Accuri™ C6 Plus. The difference in the results was either due to the high flow speed of raw water or because of not performed cleaning of the system.

After conducted analyses of the untreated raw water (at the waterwork), it was decided to perform next experiment with the flow speed of raw water adjusted at less than 25 litres per hour. This decision was based on the assumption that the flow speed of the untreated raw water was too high for performing measurements at the waterwork. The results are shown in Figure 50 and Figure 51.

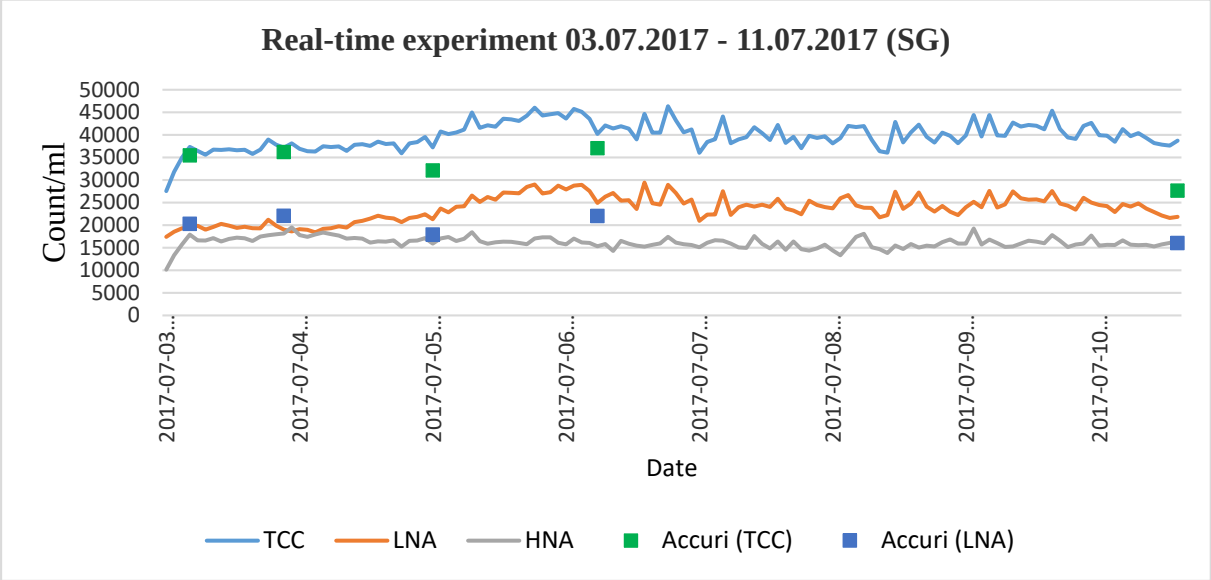

Figure 48: Results of real-time experiment 03.07.2017 - 11.07.2017 (SG)

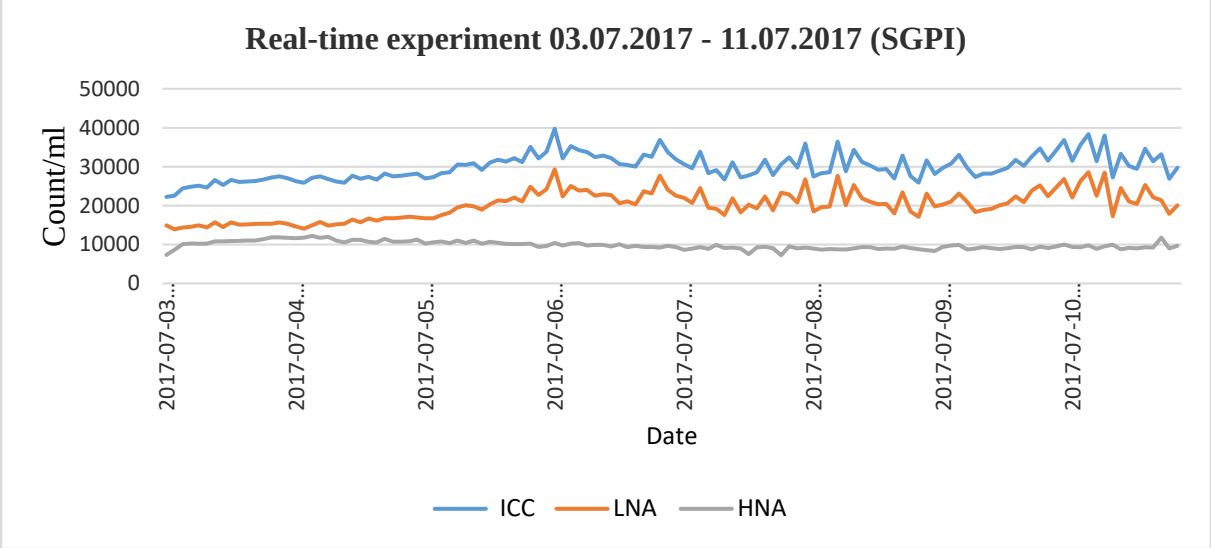

Figure 49: Results of real-time experiment 03.07.2017 - 11.07.2017 (SGPI)

Before the following experiment started the system had been cleaned with cleaning solution. The flow speed of untreated raw water was adjusted at less than 25 litres per hour. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus. The results of one week of regular measurements with SG and SGPI of the untreated raw water (at the waterwork) are shown in Figure 50 and Figure 51.

Figure 50 shows an increase of TCC and LNA in the beginning of the experiment, due to the system cleaning. Afterwards, during the experiment the growth in TCC rose from 30000 to

80000 counts/ml and from 16000 to 50000 counts/ml in LNA. This steadily growing was because of the low flow speed of untreated raw water. Water in the hose stagnated, and ensuring perfect conditions for the growth of cells. Results for measurements with SGPI stain are represented in Figure 51.

Figure 51 and Figure 50 alike showed a steady growth of cells in ICC and LNA. The linear trendline for ICC started at 23000 counts/ml and ended at 56000 counts/ml (the standard deviation was less than 5%). For LNA the count/ml increased from 13000 to 40000 cells. This research shows that low flow speed can lead to the contamination of water in hose and measurements would not represent the real number of cells. According to the obtained results it was decided to set the flow speed of water at 25 L/hour, for avoiding growth of cells in a hose. Due to the contamination of water in the hose it was decided to install a new autoclaved hose and to perform cleaning application with cleaning solution every 24 hours. The results are shown in Figure 52 and Figure 53.

The reference measurements with BD Accuri™ C6 Plus (shown in Figure 50) were conducted on the day 0, 2, 3, 5 and day 6. TCC on the day 0 was similar to the values obtained by OBA. The next results of the reference measurements were different to the OBA. The number of detected cells by OBA was higher in comparison to reference measurements with BD Accuri™ C6 Plus. The difference in the obtained results was due to not performed cleaning of the system.

After this experiment, it was decided to conduct the real-time experiment with cleaning solution cleaning. The results are shown in the chapter: 5.2.2.2 Real-time experiments with every 24 hours cleaning solution cleaning.

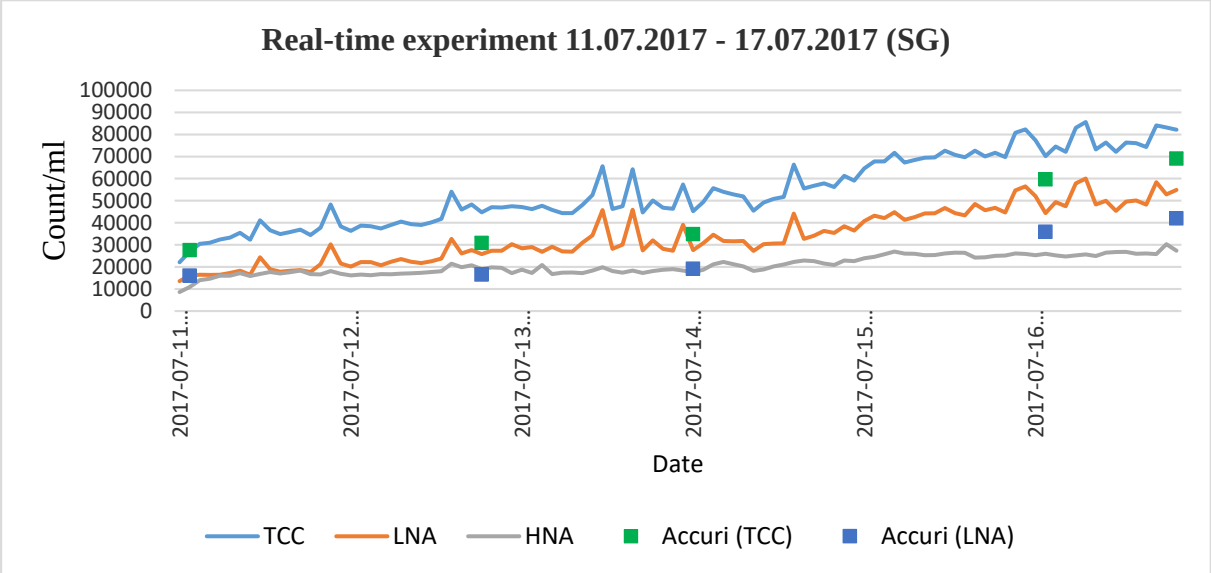

Figure 50: Results of real-time experiment 11.07.2017 - 17.07.2017 (SG)

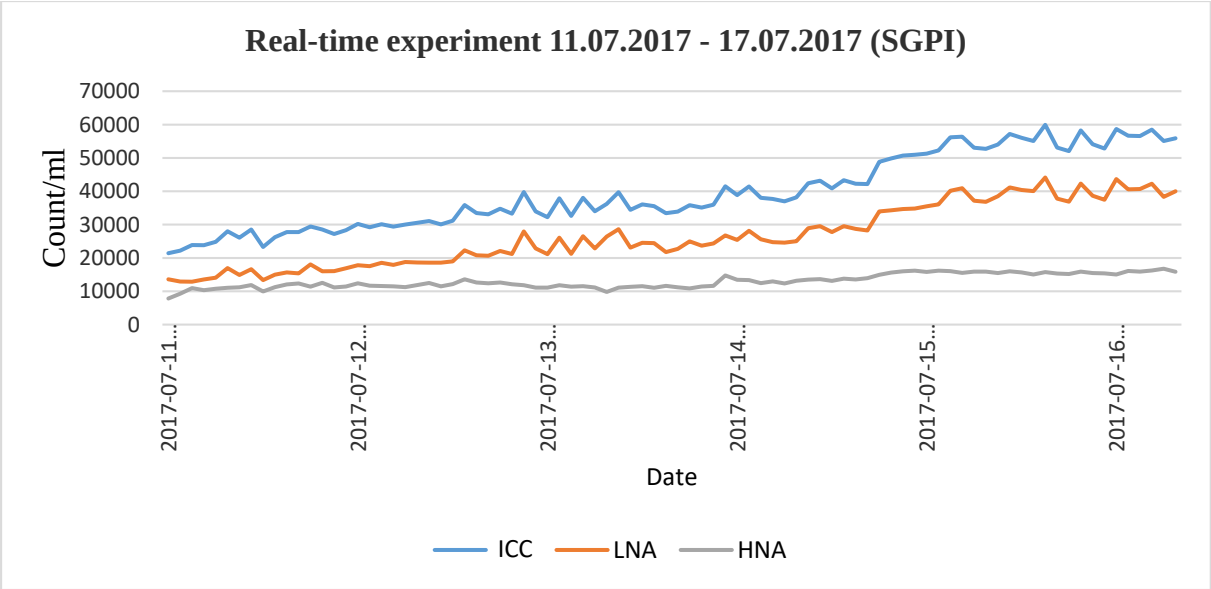

Figure 51: Results of real-time experiment 11.07.2017 - 17.07.2017 (SGPI)

5.2.2.2 Real-time experiments with every 24 hours cleaning solution cleaning

For subsequent research, the system was cleaned with cleaning solution, and new autoclaved hose was installed. The flow speed of untreated raw water was adjusted to 25 litres per hour. During the whole experiment cleaning with cleaning solution was done every 24 hours. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus. The results of one week of regular measurements with SG and SGPI of the untreated raw water (at the waterwork) are shown in Figure 52 and Figure 53.

Figure 52 shows results for one-week measurements with SG stain and with cleaning with cleaning solution every 24 hours. At the beginning of analysis an increase of cells was observed because of the cleaning of the system. Afterwards stable results were seen. TCC for water varied from 65000 to 75000 counts/ml (the standard deviation was less than 5%). LNA values during the whole experiment fluctuated from 40000 to 47000 counts/ml. Also Figure 52 shows every cleaning step. Cleaning was done every 24 hours, and the number of cells also was going down. Cleaning solution influenced results and cut down the real number of cells after each cleaning (according to the obtained results that are shown in chapter: 5.1.4.2 Effect of system cleaning with cleaning solution). On average, after cleaning with cleaning solution the first three measurements did not represent the real number of cells. The cleaning with cleaning solution was an essential tool for preventing the possibility of growth of bacteria in a hose as shown in Figure 50. Results for measurements with SGPI are shown in Figure 53.

Figure 53 and Figure 52 alike the same changes were observed. After each cleaning with cleaning solution the real number of cells was reduced. ICC for untreated raw water was stable and varied from 49000 to 58000 counts/ml (the standard deviation was less than 5%). LNA values fluctuated from 33000 to 39000. Results showed that cleaning every 24 hours was a useful tool for having no increase in the number of bacterial cells as was observed by experiment with the lower flow speed in Figure 51.

The reference measurements with BD Accuri™ C6 Plus (shown in Figure 52) were conducted on a daily basis. The results of the reference measurements were similar to the OBA. The deviation between BD Accuri™ C6 Plus and OBA results was less than 5%. Results that are presented in Figure 52 showed that cleaning of the system with cleaning solution every 24 hours was good in the field research.

After conducted analyses of the untreated raw water (at the waterwork), it was decided to perform next real-time experiment with a cleaning with cleaning solution every 12 hours. This decision was due to possible pump problems at the waterwork that were expected in the next week of the experiment. The results are shown in the chapter: 5.2.2.3 Real-time experiments with every 12 hours cleaning solution cleaning.

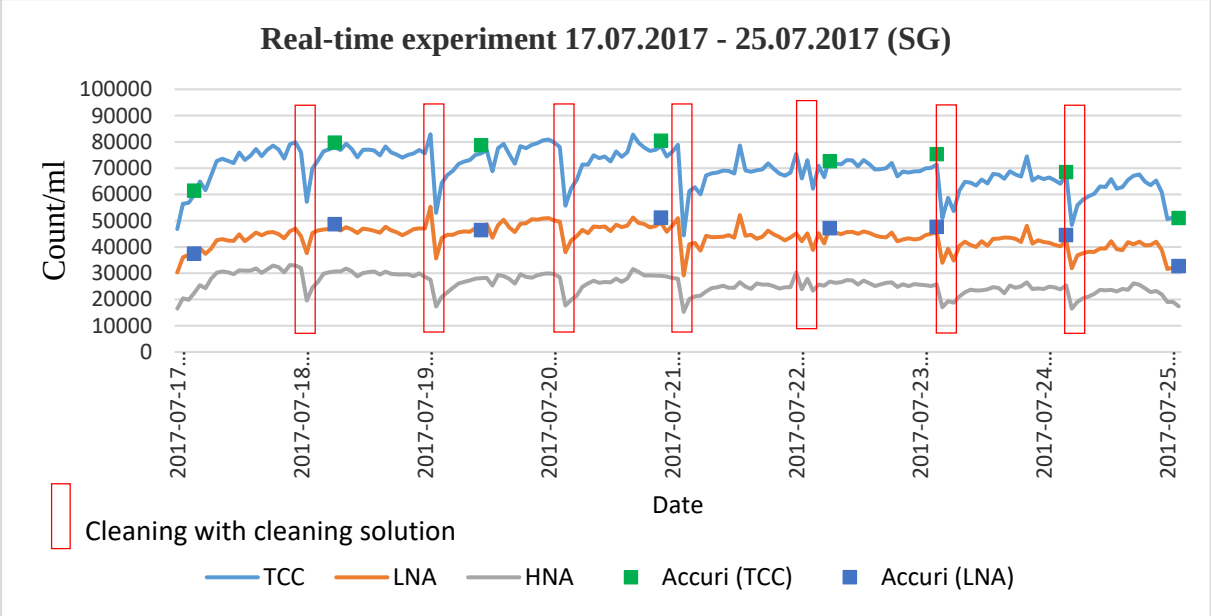

Figure 52: Results of real-time experiment 17.07.2017 - 25.07.2017 (SG)

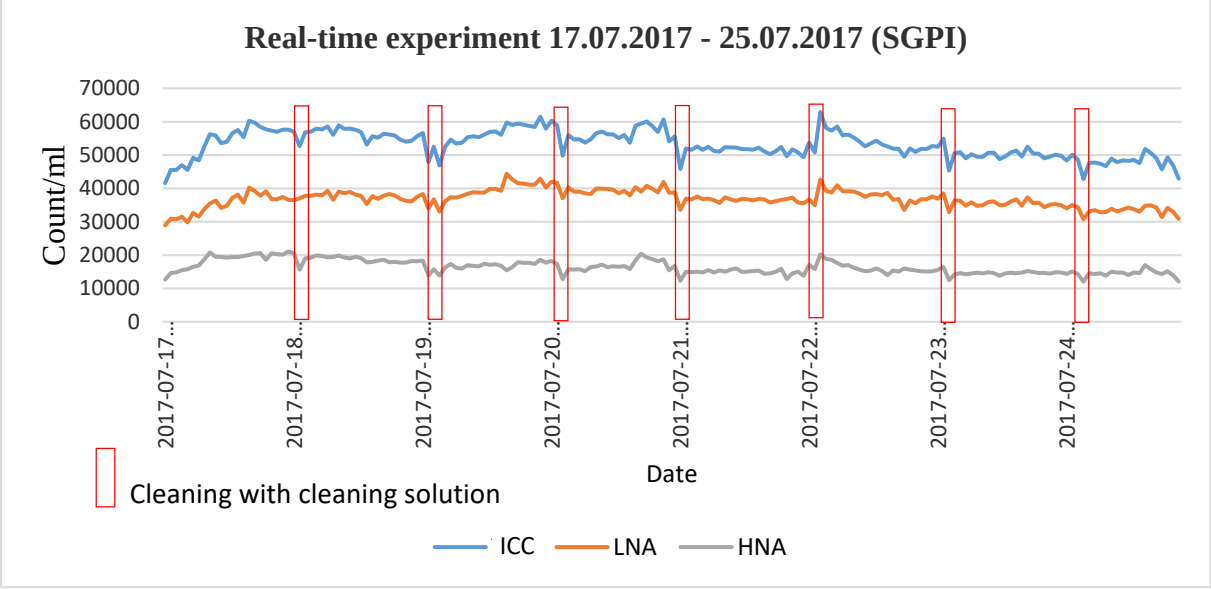

Figure 53: Results of real-time experiment 17.07.2017 - 25.07.2017 (SGPI)

5.2.2.3 Real-time experiments with every 12 hours cleaning solution cleaning

After the experiments with the cleaning of the system every 24 hours were finished (Figure 52 and Figure 53) another one was initiated but with a cleaning with cleaning solution every 12 hours. Before the following experiment was started, the system was cleaned. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus. The results of one week of regular measurements with SG and SGPI of the untreated raw water (at the waterwork) are shown in Figure 54 and Figure 55.

Figure 54 shows an increase in the number of cells in the beginning of analysis, because of the system cleaning that had been done prior to the experiment. The first two days showed a considerable amount of cells in TCC, LNA and HNA. Total cell count rose from 46000 counts/ml up to 100000 count/ml. LNA varied from 30000 to 64000 counts/ml. The values at that time were much more prominent in comparison to other values. The reason for this growth was the change of the water pump at the waterwork.

Afterwards, the quality of untreated raw water for the next two days stabilised, and the values for TCC and LNA reduced. The average for TCC was 40000 counts/ml and 18000 count/ml for LNA. Further problems with the water pump occurred at the waterwork which triggered changes in cell count. TCC rose up to 108000 counts/ml and LNA up to 63000 count/ml. The problem with the water pump lasted one day.

Next day of measurements showed rather stable results in TCC and LNA. Average value for TCC was 33000 counts/ml and 21000 counts/ml for LNA. Cleaning with cleaning solution was done every 12 hours, and the consequences of the cleaning were observed. Likewise, in the research done with cleaning every 24 hours (results are listed in Figure 52 and Figure 53) a decrease of cell number directly after cleaning with cleaning solution was observed (according to the obtained results that are shown in chapter: 5.1.4.2 Effect of system cleaning with cleaning solution). The first three measurements after cleaning did not represent the real value of cells. Results with SGPI stain are shown in Figure 55.

In Figure 55 and Figure 54 the same changes in water quality were noticed. On the first two days, the growth in ICC stood at 75000 counts/ml and 55000 counts/ml in LNA. The subsequent two days provided stable results. ICC average was 27000 counts/ml and 18000 counts/ml in LNA.

Afterwards, new problems with the water pump at the waterwork arose which lasted one day. Consequently, ICC rose up to 83000 counts/ml, and LNA up to 55000 count/ml at the same time. After the waterpump problems were solved the results remained stable until the end of the experiment. The ICC average was 24000 counts/ml, and LNA's 18000 counts/ml.

The reference measurements with BD Accuri™ C6 Plus (shown in Figure 54) showed similar to the OBA results. The deviation between BD Accuri™ C6 Plus and OBA results was less than 10%. Results that are presented in Figure 54 showed that cleaning of the system with cleaning solution every 12 hours in the event of problems with the water pump was a good solution in the field research.

To prevent further potential distortion of experimental results due to malfunctioning waterpump cleaning every 12 hours was an essential tool for this research. According to the obtained results it was decided to perform a cleaning with cleaning solution every 12 hours in next real-time experiment. This decision was aimed to check the cleaning application at the waterwork for a more extended period of time. The results are shown in Figure 56 and Figure 57.

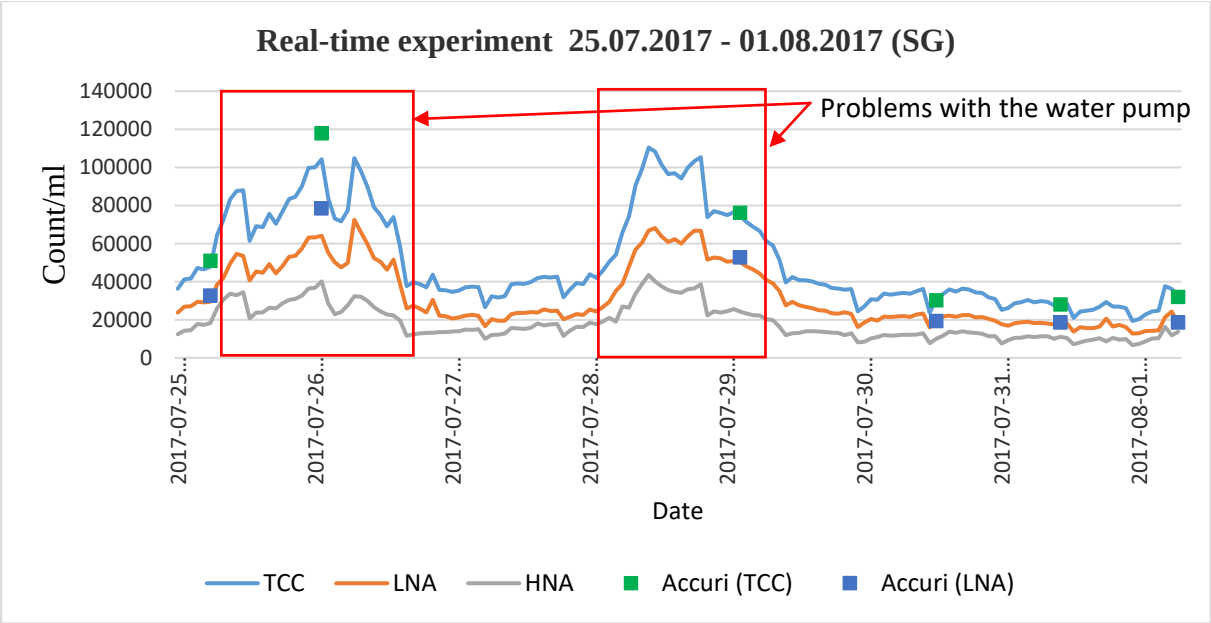

Figure 54: Results of real-time experiment 25.07.2017 - 01.08.2017 (SG)

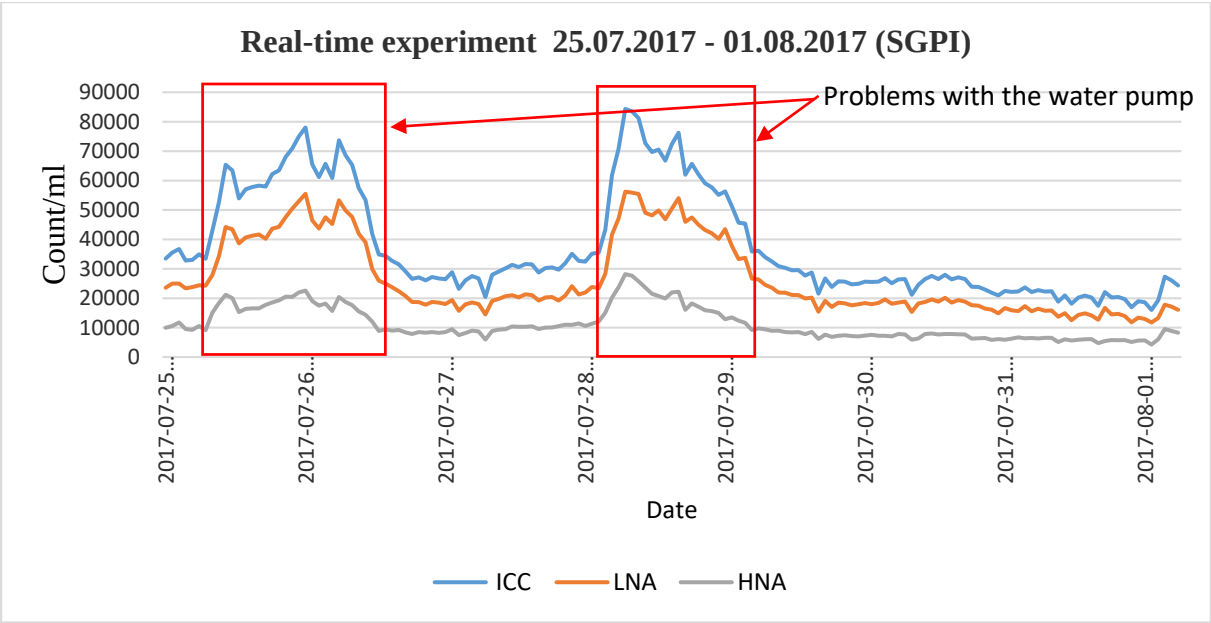

Figure 55: Results of real-time experiment 25.07.2017 - 01.08.2017 (SGPI)

The research with the cleaning of the system every 12 hours of measurements was continued to gather more data. Before another experiment was started, the system had been cleaned. The results of one week of regular measurements with SG and SGPI of the untreated raw water (at the waterwork) are shown in Figure 56 and Figure 57.

Figure 56 shows no changes in water quality during the whole time of the experiment. Only on the fifth day of the experiment differences in the number of cells were detected. An increase in total cell counts from 90000 to 180000 counts/ml was noticed. Shortly after the number of cells stabilised and returned to previous levels. Cleaning every 12 hours provided reliable and representative results, but similar to previous experiments it influenced the first three measurements (according to the obtained results that are shown in chapter: 5.1.4.2 Effect of system cleaning with cleaning solution). Results with SGPI stain are shown in Figure 57. The reference measurements with BD Accuri™ C6 Plus (shown in Figure 56) were similar to the OBA results. The deviation between BD Accuri™ C6 Plus and OBA results was less than 10%.

Figure 57 and Figure 56 displayed no visible changes in the number of bacteria for the first five days of measurements. Afterwards, like in Figure 56 changes in the total amount of bacteria were detected, because of the pump change at the waterwork. The spikes in cell counts can be observed in Figure 57. These spikes occurred after each cleaning that took place every 12 hours. Cleaning was essential for obtaining stable data, but every first three measurements did not represent the real amount of bacteria. Due to the obtained results it was decided to perform a cleaning with cleaning solution every 16 hours in next real-time experiment. The results are shown in the chapter: 5.2.2.4 Real-time experiments with every 16 hours cleaning solution cleaning.

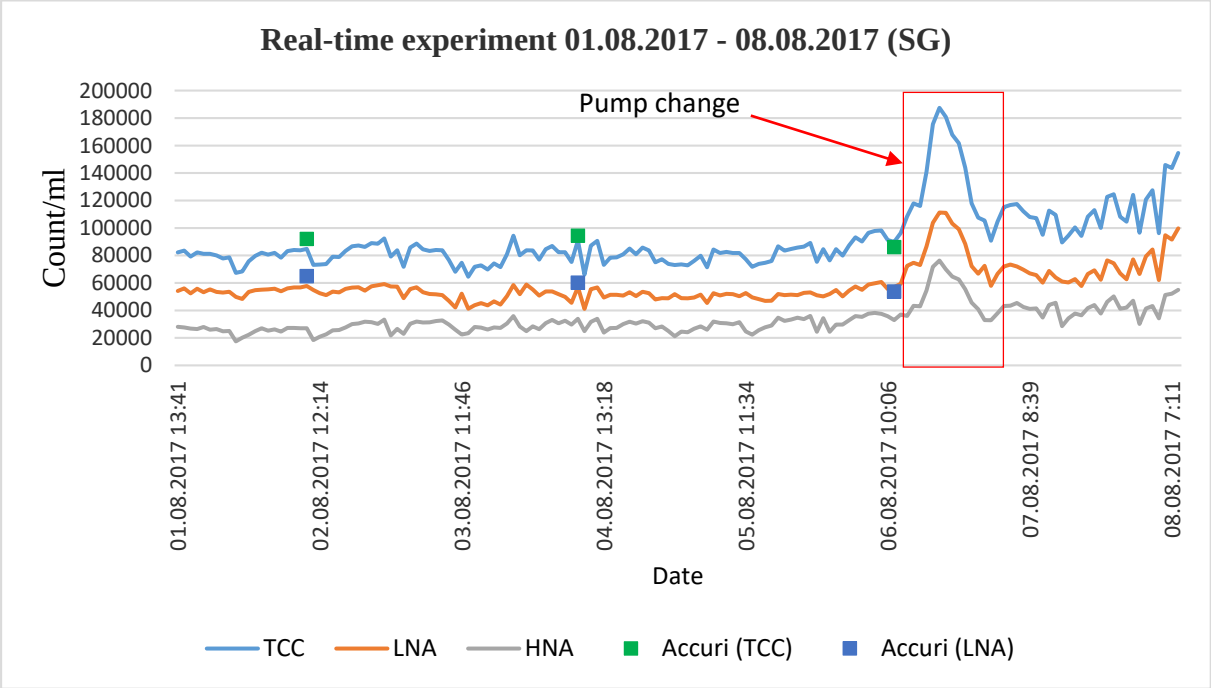

Figure 56: Results of real-time experiment 01.08.2017 - 08.08.2017 (SG)

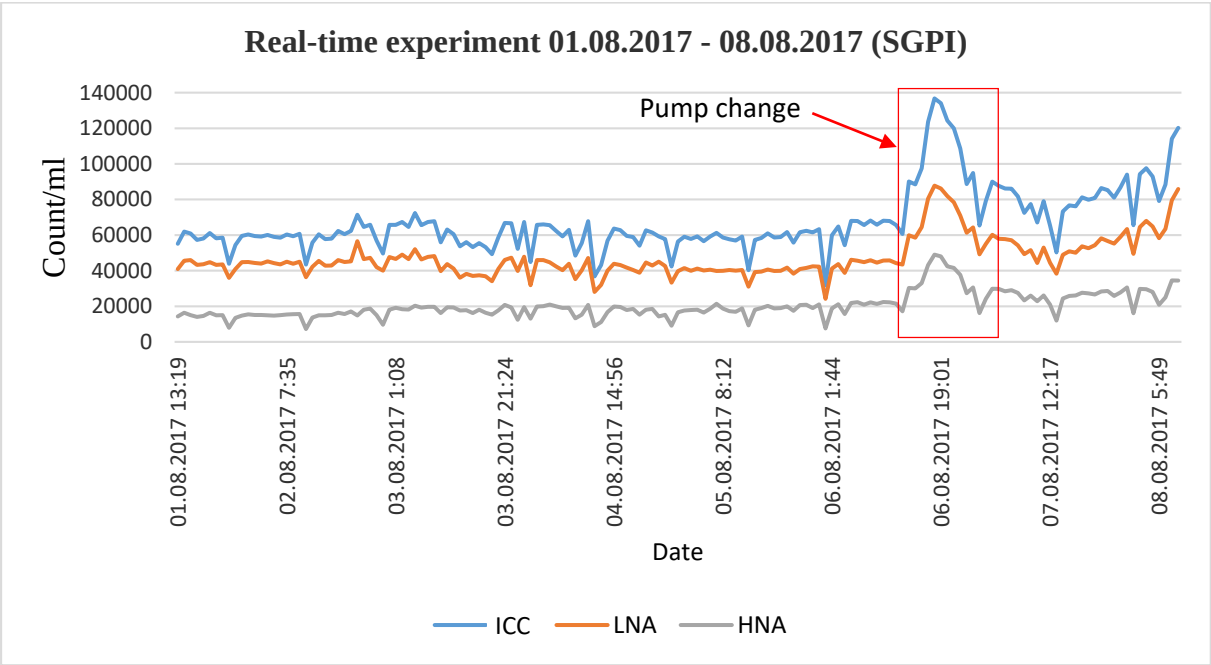

Figure 57: Results of real-time experiment 01.08.2017 - 08.08.2017 (SGPI)

5.2.2.4 Real-time experiments with every 16 hours cleaning solution cleaning

The next step of the research was to measure water in a flow with cleaning every 16 hours. Before this was carried out the system was cleaned with cleaning solution. Reference measurements were performed with the flow cytometer BD Accuri™ C6 Plus and CyFlow® Cube 6. The results of one week of regular measurements with SG and SGPI of the untreated raw water (at the waterwork) are shown in Figure 58 and Figure 59.

In Figure 58 and Figure 59 display a slight decrease in the number of bacteria in the first four days of the experiment. Afterwards, the water source at the waterwork was changed and considerable changes in water quality were observed. The TCC remarkably increased from 30000 to 135000 counts/ml for measurements with SG and the ICC increased from 26000 to 102000 counts/ml for measurements with SGPI. After the shift in the number of cells was observed the number of bacteria slightly decreased to the point of stabilisation. The spikes similar to previous measurements were observed (alike in Figure 52 – Figure 57). Cleaning application every 16 hours showed reliable and representative data.

The reference measurements with BD Accuri™ C6 Plus and CyFlow® Cube 6 (shown in Figure 58) were similar to the OBA results. The deviation between BD Accuri™ C6 Plus and OBA results was less than 10%. The deviation between CyFlow® Cube 6 and OBA results was less than 5%. These obtained values show excellent compatibility in the results.

The spikes that are seen in Figure 52 – Figure 59 were obtained after each system cleaning with the cleaning solution. This statement confirms the results that are represented in the Figure 34. This means that after system cleaning was performed, some amount of cleaning solution remained in the equipment. Residues of cleaning solution that left behind in the system after cleaning minimise the real amount of bacterial cells (according to the results that are shown in chapter: 5.1.4.2 Effect of system cleaning with cleaning solution). Due to the obtained results, it was decided to perform cleaning with cleaning solution every 16 hours for real-time experiments.

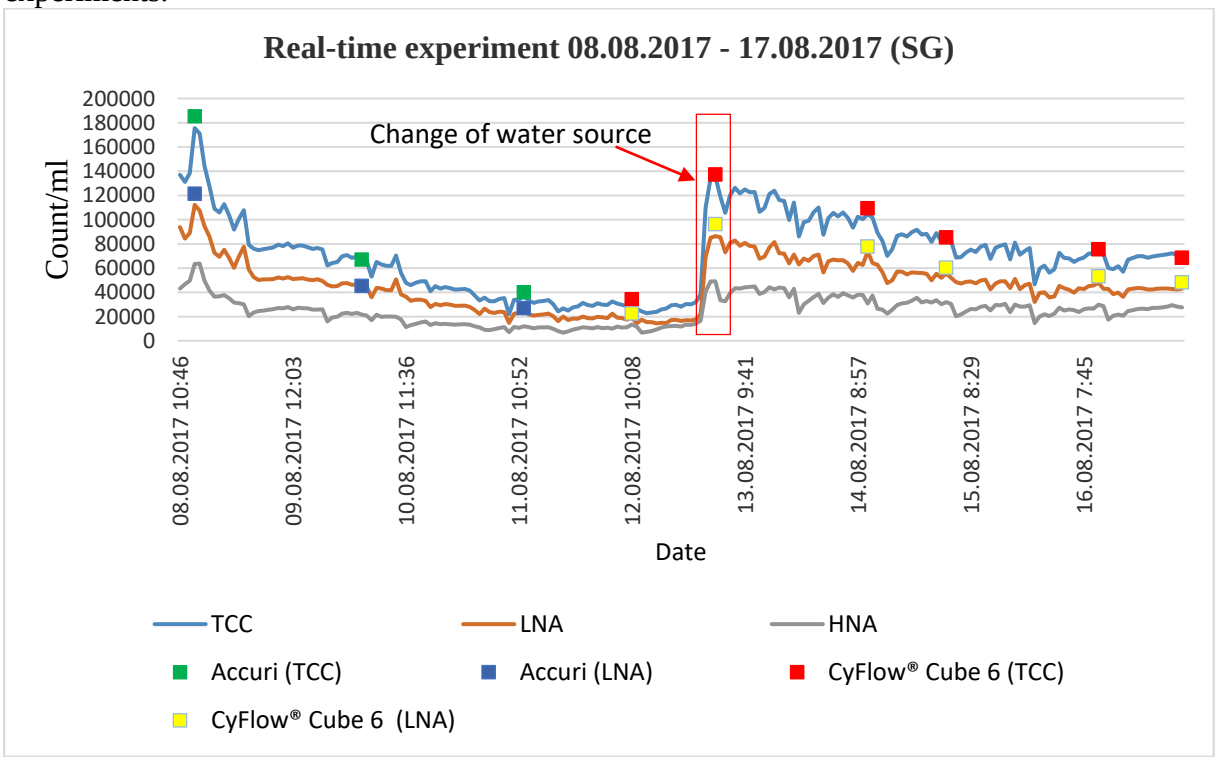

Figure 58: Results of real-time experiment 08.08.2017 - 17.08.2017 (SG)

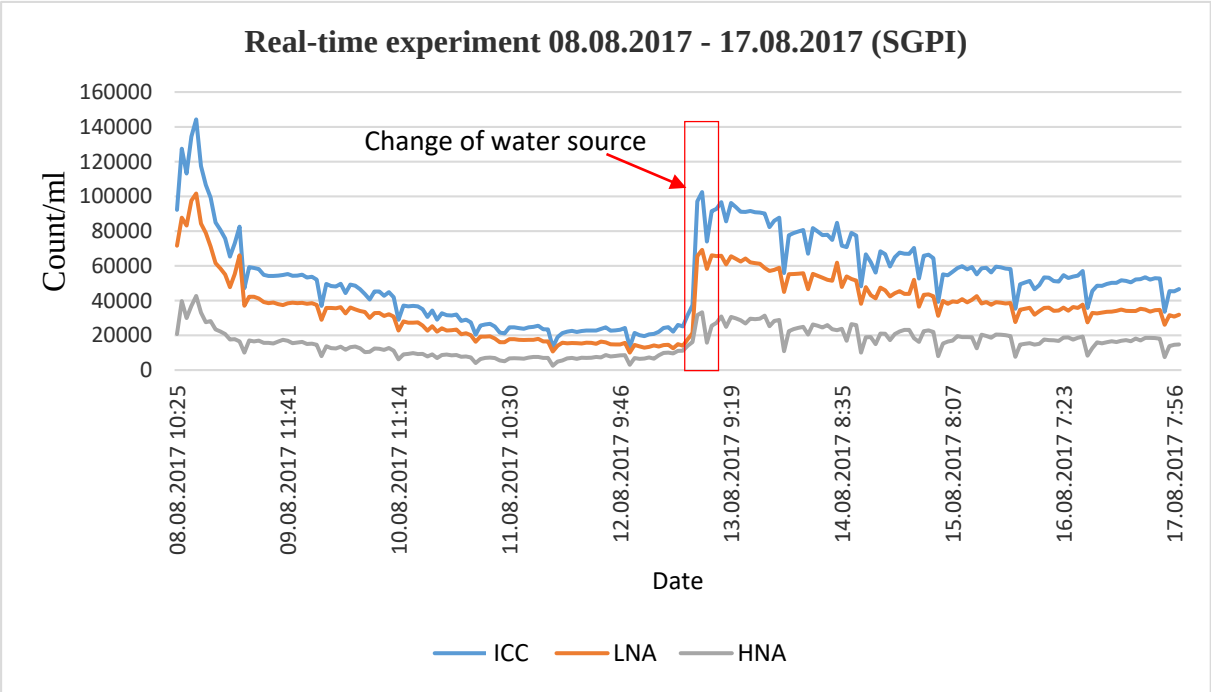

Figure 59: Results of real-time experiment 08.08.2017 - 17.08.2017 (SGPI)

5.2.3 TCC results in real-time experiments

The monitoring of raw water at the waterwork was done in real-time with an automated sampling system. The experiment was done 24 hours a day, seven days a week (24/7) to observe changes in the water quality of the water. Untreated raw water was directly analysed from the flow at the waterwork, and reference measurements were performed in the laboratory with the flow cytometer BD Accuri™ C6 Plus. Comparison of reference measurements which were done in the laboratory with online measurements which were performed in the field (at the waterwork) is shown in Figure 60.

Figure 60 shows a good agreement of reference measurements done in the laboratory with measurements which were done in real-time. Few deviations that are seen in Figure 60 were due to the influence of water samples transportation to the laboratory. The regular decrease of total cell numbers by real-time measurements in the flow was due to the performed cleaning with cleaning solution (according to the obtained results that are shown in the chapter: 5.1.4.2 Effect of system cleaning with cleaning solution). According to the obtained results in the real-time experiment, the application of online-flowcytometry at the waterwork showed comparable results to the reference measurements which were conducted in the laboratory. The advantage of real-time flow cytometry compared to flow cytometry in the laboratory is faster in obtaining of results and also saving money for sampling.

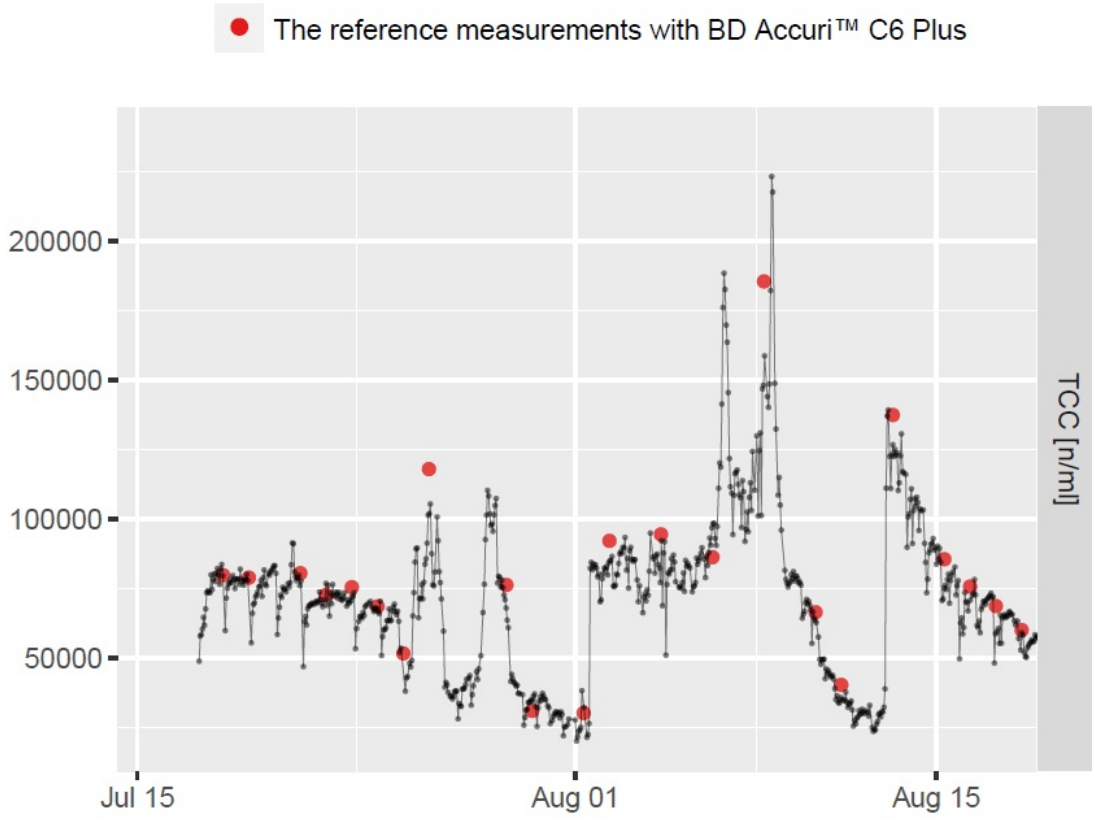

Figure 60: Comparison between the lab and the real-time measurements

5.2.4 Comparison of TCC and ICC results in real-time experiment

Changes in microbiological parameters such as TCC and ICC were estimated in real-time experiments (results are shown in the chapter: 5.2.2 Research mode real-time experiment). Comparison of the measurements during the timespan of research conducted with SG (for TCC) and SGPI (for ICC) is shown in Figure 61.

Figure 61 displayed the changes of untreated raw water quality in real-time research. The measurements obtained with SG for estimating TCC were comparable to the measurements with SGPI for estimating ICC. According to the obtained results, the simultaneous application of SG and SGPI stains in the real-time experiment does not affect the result.

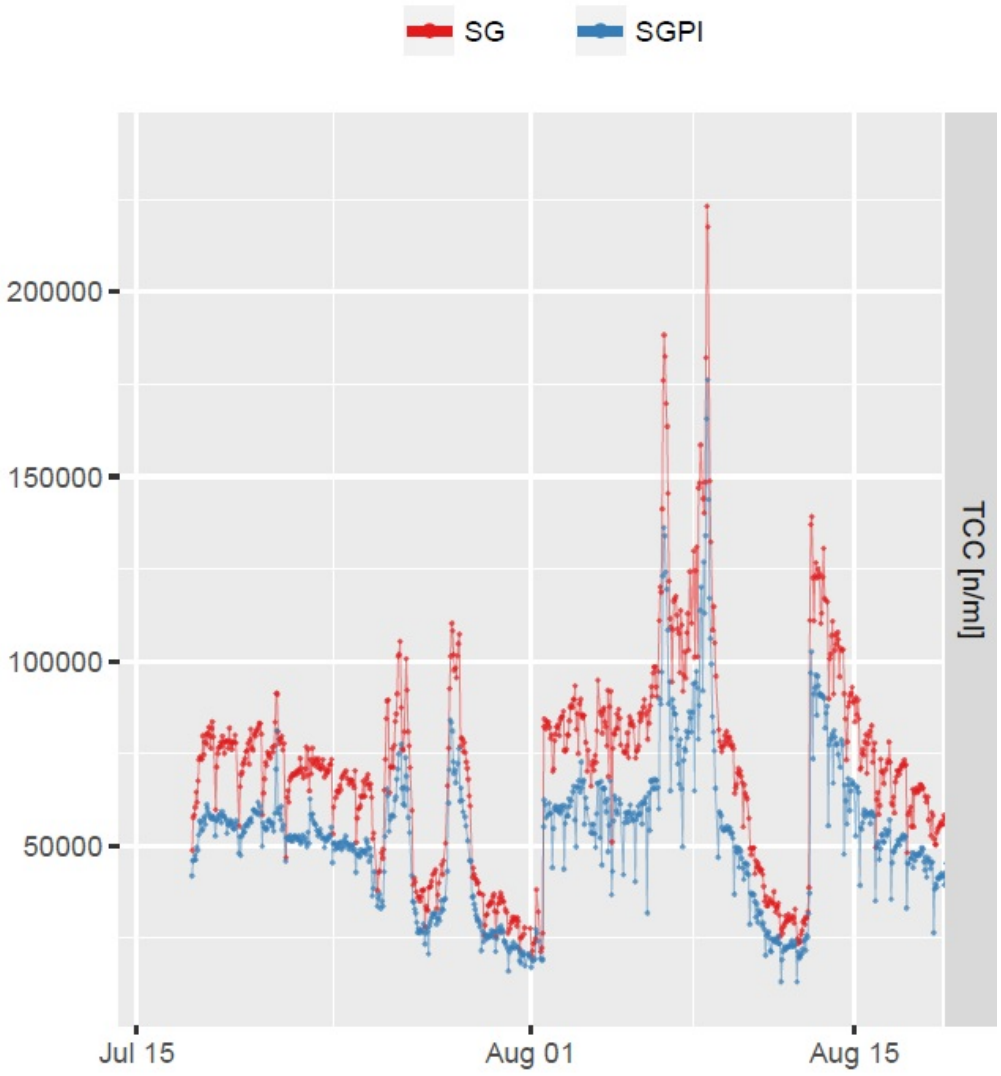

Figure 61: Comparison of SG and SGPI results in real-time experiment

6 Conclusion and outlook

Data obtained from the measurements of different water samples conclude the following:

- The incubation time for stains SG and SGPI are different. The incubation time for SG was fixed at 5 minutes and for SGPI at 10 minutes. The incubation temperature for both stains was 40 °C. These conditions are optimal for applying the OBA in the research to get stable results.
- The stains stored in OBA at 15 °C could be used for no longer than two months. This decision was based on obtained results with freshly prepared and stored stains. Stains were regularly changed for gathering representative data about the real amount of cells in water samples.
- Stand trial research showed an increase of bacterial cells in UV-C processed water samples measured by OBA, but showed no growth of bacteria by reference measurements with BD Accuri™ C6 Plus. The increase in the number of cells in water samples measured by OBA was because of the not performed cleaning of the equipment. The cleaning application with pure water which was performed every 24 hours in stand trial experiments showed the better ability for measuring the growth of bacterial cells. The opportunity to measure growth of bacteria is an essential tool for applying flow-cytometer in exploring stability of water samples.
- The analysis of drinking water was done using automated sample ports that took and measured the samples every 20 minutes. This advantage makes OBA very attractive for planning different researches which are conducted to measure water samples in a short time span.
- It is recommended that the cleaning procedure in real-time with cleaning solution should be carried out every 16 hours if the water quality at the waterwork is expected to change. Cleaning of the system with a cleaning solution every 24 hours is recommended to apply if the water quality at the waterwork is stable. This cleaning is necessary for keeping the system clean and obtaining stable results. Some amount of cleaning solution remains in the equipment after the cleaning. Residues of cleaning solution that stay in the system after cleaning reduce the real amount of bacterial cells.

The application of OBA showed that online-flow cytometer could be applied not only for laboratory research but also in the field (at the waterwork). The stability of obtained results and rapid detection of bacterial cells (within 20 minutes due to automated sampling system) could expand the application of flow cytometry.

Advantages and limitations of the OBA utilization are listed in the Table 24. Limitations noted in Table 24 form the basis for future development of OBA usage in water analysis.

Table 24: OBA advantages and limitations

Application	Advantages	Limitations
General	Easy to use; Measurement within 20 minutes; Automatic sampler; Two stains usage; Two automated sample ports and one manual sample port. Incubation directly after mixing stain with sample; Internet connection.	Software problems: Not possible to select only one sample port (A or B) for doing the cleaning; In “sequence”, only two processes could be defined. It is needed to have at least four processes in “sequence”.
Stand trial	Simple determination of bacterial growth in water samples.	Cleaning solution can drip into the sample and change the results.
Real-time	Constant monitoring of water quality; Warning and alarm e-mails.	No flow speed measuring instrument; Ingress of air into the system during low or no flow.

For future studies with the Online Bacteria Analyzer the following should be done:

1. Analysing the issue of the spikes obtained after each cleaning with the cleaning solution that was seen in Figure 52 – Figure 59.
2. Integrating a flow speed measuring instrument into the sample port. This tool would be essential for applying the online-flow cytometer into the field.
3. The cleaning effect of the system should be checked for the presence of water and/or cleaning solution residues after the system cleaning.

7 Summary

This thesis aims to check the application of the online-flow cytometry in the continuous flow and the stability of obtained measurements by applying new equipment. Therefore, were established specific conditions, settings, were established to enhance usability and effectiveness of the flow cytometer in the water analysis.

In this master thesis the Online Bacteria Analyzer (OBA) produced by METANOR AG was employed for water analysis. The research was based on the information received from the literature analysis. All measurements in the lab scale and at the waterwork were done from April 2017 until December 2017. The research was conducted on different water samples with different stains. Firstly, the measurements in the lab scale were done, in order to understand the main processes taking place.

In the lab scale research, the following were carried out:

- Establishing issues about specific conditions, settings, usability and effectiveness of the flow cytometer in the water analysis;
- Calculating liquid consumption per one process (Table 4) and per one week (Table 5) of exploitation;
- Examining stain and incubation times TCC/ICC (results are shown in the chapter: 5.1.1 Stain and incubation times);
- Defining the frequency of the system cleaning (results are shown in the chapter: 4.1.2 Cleaning of the equipment);
- Estimating the influence of cleaning with pure water/cleaning solution cleaning (results are shown in the chapter: 5.1.4 Effect of system cleaning);
- Specifying measuring plot areas for different stains for further estimation of water analysis in lab scale/field research (4.1.5 Measuring plot area (Gating));
- Defining the calibration plot area and doing calibration measurements for the evaluation of new equipment (results are shown in the chapter: 4.2.3 Calibration measurements);
- Conducting analyses in stand trial research (results are shown in the chapter: 4.2.5 Test mode stand trial experiment);
- Applying the online-flow cytometer in the real-time (results are shown in the chapter: 4.2.6 Test mode real-time experiment).

After checking all the previous points in the lab scale, OBA was employed at the waterwork for analyzing different water samples.

In the field research, the following were carried out:

- Providing numerous stand trial experiments:
    1. Without pure water cleaning (results are shown in the chapter: 5.2.1.1 Stand trial experiment without cleaning);
    2. With pure water cleaning (results are shown in the chapter: 5.2.1.2 Stand trial experiment with pure water cleaning).
- Monitoring microbiological changes in drinking water. Water samples were analysed in real-time with different applications of system cleaning:
    1. Without cleaning solution cleaning (results are shown in the chapter: 5.2.2.1 Real-time experiments without cleaning);
    2. With every 24 hours cleaning solution cleaning (results are shown in the chapter: 5.2.2.2 Real-time experiments with every 24 hours cleaning solution cleaning);
    3. With every 12 hours cleaning solution cleaning (results are shown in the chapter: 5.2.2.3 Real-time experiments with every 12 hours cleaning solution cleaning);

4. With every 16 hours cleaning solution cleaning (results are shown in the chapter: 5.2.2.4 Real-time experiments with every 16 hours cleaning solution cleaning);

The results in the lab scale research:

1. It was decided to use stain B – SYBR Green prepared at BOKU for TCC determination with an incubation time adjusted at 5 minutes and to use stain A - SYBR Green propidium iodide for ICC determination with an incubation time adjusted at 10 minutes (results are shown in the chapter: 5.1.1 Stain and incubation times).
2. Stain B – SYBR Green prepared at BOKU estimated the similar amount of bacterial cells in comparison to stain C – SYBR Green prepared by METANOR©. Therefore, the stain B prepared at BOKU was used in the conducted experiments (results are shown in the chapter: 5.1.1 Stain and incubation times).
3. The used stains were no older than two months for obtaining representative data information about the real amount of cells in water samples (results are shown in the chapter: 5.1.2 Stability of stains).
4. The plot area (gate) was chosen to be comparable to another applied flow cytometers: BD Accuri™ C6 Plus and CyFlow® Cube 6 (results are shown in the chapter: 5.1.3 Calibration measurements).
5. The cleaning application with pure water or with cleaning solution underestimated the real amount of bacterial cells after each cleaning application and influenced the results.

The results in the field research:

1. The stand trial experiments were performed under the cleaning application every 24 hours due to the possible contamination of the equipment (results are shown in the chapter: 5.2.1 Research mode stand trial experiment).
2. The application of the online-flow cytometer at the waterwork was an essential tool for estimating changes in the number of cells in water samples.
3. Cleaning was essential for obtaining stable data, but every first three measurements did not represent the real amount of bacteria, because of the residues of cleaning solution that stay in the system after each cleaning application which reduce the real amount of bacterial cells. Cleaning application every 16 hours with cleaning solution in the real-time experiments showed reliable and representative data (results are shown in the chapter: 5.2.2 Research mode real-time experiments).
4. Cleaning of the system with cleaning solution every 24 hours is recommended to apply if the water quality at the waterwork is stable (results are shown in the chapter: 5.2.2.2 Real-time experiments with every 24 hours cleaning solution cleaning).
5. The stability of all obtained measurements showed that the flow cytometer could be applied in the raw and drinking water analysis.

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9 Appendix

9.1 OBA standard operating procedures (SOPs)

9.1.1 Activation

For OBA activation the following steps are necessary:

1. Checking all connections;
2. Switching on of OBA connection pattern breaker. METANOR© software automatically started;
3. Power on (METANOR© software);
4. Checking device and fluid containers status:
   - ✓ Stain (15.0/15.0)
   - ✓ Reactor (40.0/40.0)
   - ✓ Laser (27.2/27.2)

Figure 62: OBA device and fluid containers status

All light signals were green. If one of these parameters was not fulfilled, then the system was restarted.

9.1.2 Stain change application

After the preparation of the stain the following steps were taken:

1. Chosing one of the stain ports (A or B) for placing the stain;
2. Disconnecting stain container from stain-hose;
3. Installing stain-hose in 15 ml plastic tube;
4. Selecting HOME button in Main Menu (General);
5. Selecting the task category Built-in;
6. Selecting the task: Clean Stain Port (A or B) (Duration: ≈5 min);
7. Starting Task with start-button;
8. In progress shown overall and remaining time. Cleaning finished when green tick appeared in history;
9. Disconnecting stain-hose from 15 ml plastic tube;
10. Connecting stain-hose with prepared stain (A or B) that was already filled in 100 ml plastic OBA container.
11. Bringing in software information about the level of stain container, which was filled. For this step in Administration Menu the Settings button was used.
12. In Settings was shown the current volume of liquid containers. Current volume of stain containers (A or B) was given in liters;
13. The stain was ready to use. The prepared stain was used/stored no longer than six months at 15 °C. First measurements (2 -3 measurements) after stain changing were irrelevant.

9.1.3 Cleaning application

For general cleaning application such steps in OBA software were done:

1. Selecting HOME button in Main Menu (General);
2. Selecting the task category Clean;
3. Selecting the task:
   - Pure Water BOKU (Duration: ≈22 min). Used for cleaning after several measurements (10 - 20 Measurements).
   - Cleaning Solution BOKU (Duration: ≈38 min). Used for cleaning when the system was not clean enough.
4. Starting Task with start-button;
5. In progress shown overall and remaining time. Cleaning finished when green tick appeared in history.

By single port cleaning application, washing was done with pure water and cleaning solution. Operation steps in OBA software:

1. Selecting HOME button in Main Menu (General);
2. Selecting the task category Built-in;
3. Selecting the task: Clean Single Port (Duration: ≈5 min)
4. Starting the task with start-button;
5. In progress shown overall and remaining time. Cleaning finished when green tick appeared in history.

Remarks, current problems and recommendations by cleaning application

For setting different parameters of cleaning in OBA software such steps were done:

1. Selecting “Clean System” button in Main Menu;
2. Selecting “Clean Type”. If selected the “Pure Water” clean, then Follow-UP Clean step was not required. For Clean Type “Cleaning solution” the Follow-Up Clean should be done with pure water.

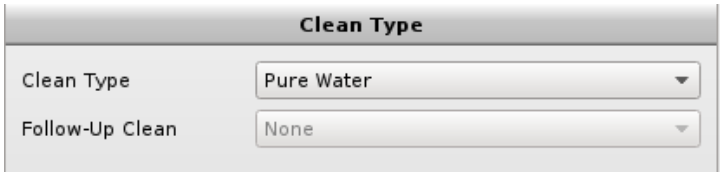

Figure 63: Clean type by OBA

By “Cleaning Ports” inset pull and push speed can be changed. It is recommended to set pull and push on max level, for having good and faster cleaning.

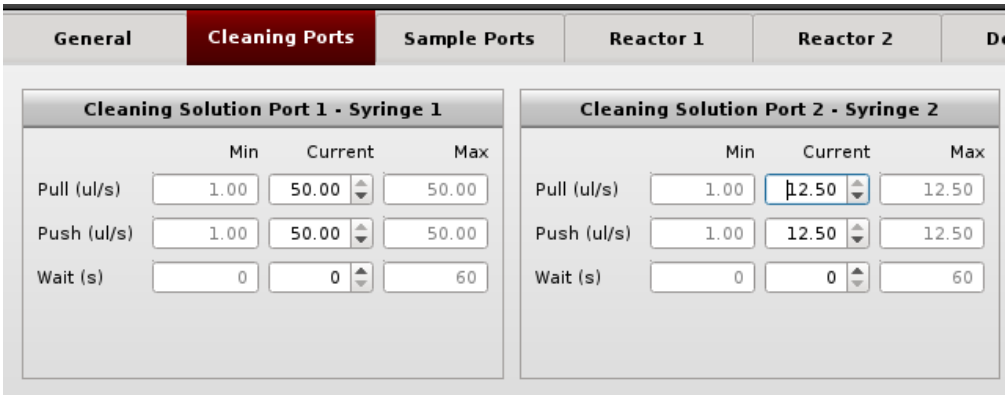

Figure 64: Cleaning ports inset by OBA

By “Sample Ports” inset many parameters can be changed. Sample Port cleaning is very important for future measurements, because all samples that would be measured in real-time would be performed by Sample Ports A or B. This cleaning can influence results by setup settings.

It is recommended to adjust “Pull and push speed” at max level, for having intensive cleaning of “Sample Ports”.

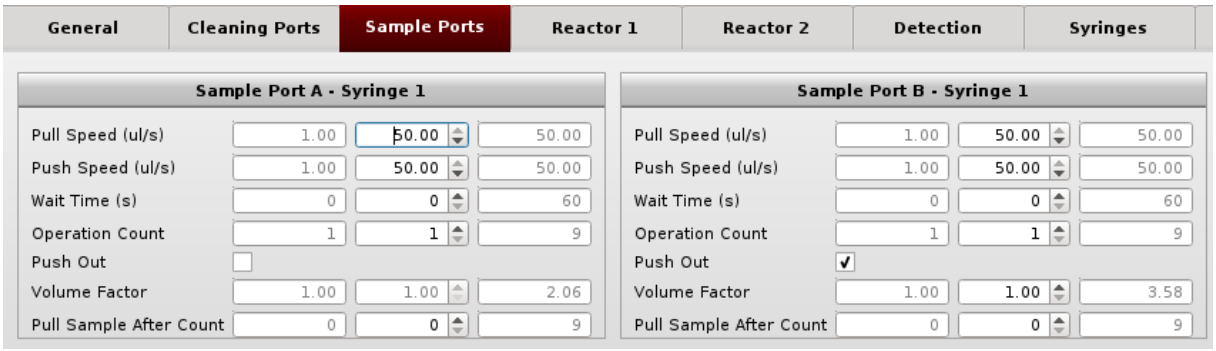

Figure 65: Sample ports inset by OBA

“Operation Count” allowed the choice between “1” and “9”. By “Operation count” the system pushes cleaning solution to the sample port and afterwards pulls the cleaning solution into the waste. It was recommended to set “Operation Count” at 1, for having minimal influence on measuring.

By “Pull Sample After Count” the system pulls the cleaning solution from 1 to 9 times to sample port. It is recommended to set the “Pull Sample After Count” at “0” for preventing discharge of cleaning solution in the water sample.

After the system cleaning with cleaning solution, the first three results did not represent the real amount of bacterial cells. This was due to the dilution of the water sample with residues of cleaning solution. After the cleaning with “Cleaning solution”, it was recommended to perform extra cleaning with “Pure water cleaning”.

9.1.4 Measure application

For sample measurements the following steps in OBA software were done:

1. Selecting HOME button in Main Menu (General);
2. Selecting the task category Process;
3. Selecting the task: SampleA-StainA (Duration: ≈18 min);
4. Starting the task with start-button;
5. Typing the name of the sample:
6. In progress showed the overall and remaining time. Cleaning finished when a green tick appeared in history.

For sample measurements with a single port steps such as those below were done in OBA software:

1. Selecting HOME button in Main Menu (General);
2. Selecting the task category Process;
3. Selecting the task: Single Port Measurement (Duration: ≈8 min).
4. Starting the task with start-button;
5. Typing the name of the sample:
6. In progress showed the overall and remaining time. Cleaning finished when a green tick appeared in history.

For pure water canister measurements, the following steps in OBA software were done:

1. Selecting HOME button in Main Menu (General);
2. Selecting the task category Process;
3. Selecting the task: Pure Water canister measurement (Duration: ≈17 min).
4. Starting the task with start-button;
5. Typing the name of the sample:
6. In progress showed the overall and remaining time. Cleaning finished when a green tick appeared in history.

9.1.5 Calibration application

Applied only by using a single port. Afterwards, “Clean Single Port” cleaning is necessary. There are two types of calibrations with beads:

- Calibration Beads (used for one peak calibration beads);
- 8-Peak Validation Beads (used for 8-peak calibration beads).

For both types of calibration, the following steps in OBA software were done:

1. Selecting HOME button in Main Menu (General);
2. Selecting the task category Built-in;
3. Selecting the task: Calibration Beads or 8-Peak Validation Beads (Duration: ≈8 min)
4. Starting the task with start-button;
5. In progress showed the overall and remaining time. Cleaning finished when a green tick appeared in history;
6. Afterwards, system cleaning was necessary. Selecting HOME button in Main Menu (General);
7. Selecting the task category Built-in;
8. Selecting the task: Clean Single Port (Duration: ≈5 min);
9. Starting the task with start-button;
10. In progress showed the overall and remaining time. Cleaning finished when a green tick appeared in history.

9.1.6 Sequence application

For a sequence definition listed steps in OBA software were done:

1. Selecting Sequence button in Main Menu (Definition);
2. Selecting “+” button for a new sequence creation;
3. Typing the name of the sequence in “General” window;
4. Selecting sequence settings in “Process/Clean/Meta-Analysis”. Steps are listed below:

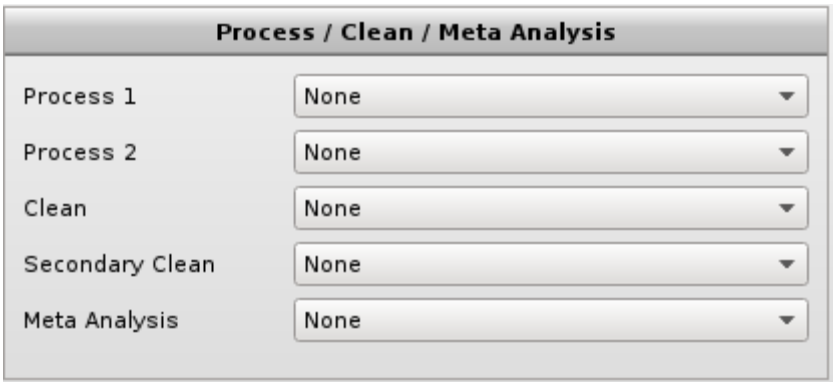

Figure 66: Sequence settings in process, clean, meta-analysis by OBA

5. Selecting one process from templates or creating a new one. Selecting at least one process in Process 1 and Process 2 categories.
6. Selection of cleaning types in clean/secondary clean categories.
7. Seting the time:
    - “Execution time” (automatically);
    - Selecting interval between measurements in “Interval (m)”;
    - Selecting cleaning interval in “Clean after intervals”.

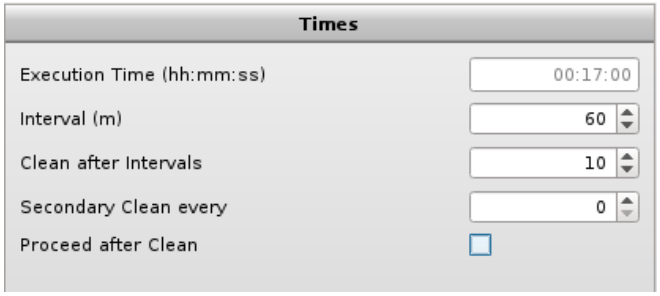

Figure 67: Sequence time setting by OBA

8. Saving the sequence with “Save” button;
9. Selecting HOME button in Main Menu (General)
10. Selecting the task category Sequence;
11. Selecting the task: Sequence;
12. Starting the task with start-button;
13. Typing the name of the sample:
14. In progress showed the overall and remaining time. Cleaning finished when a green tick appeared in history.

10 Curriculum Vitae

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