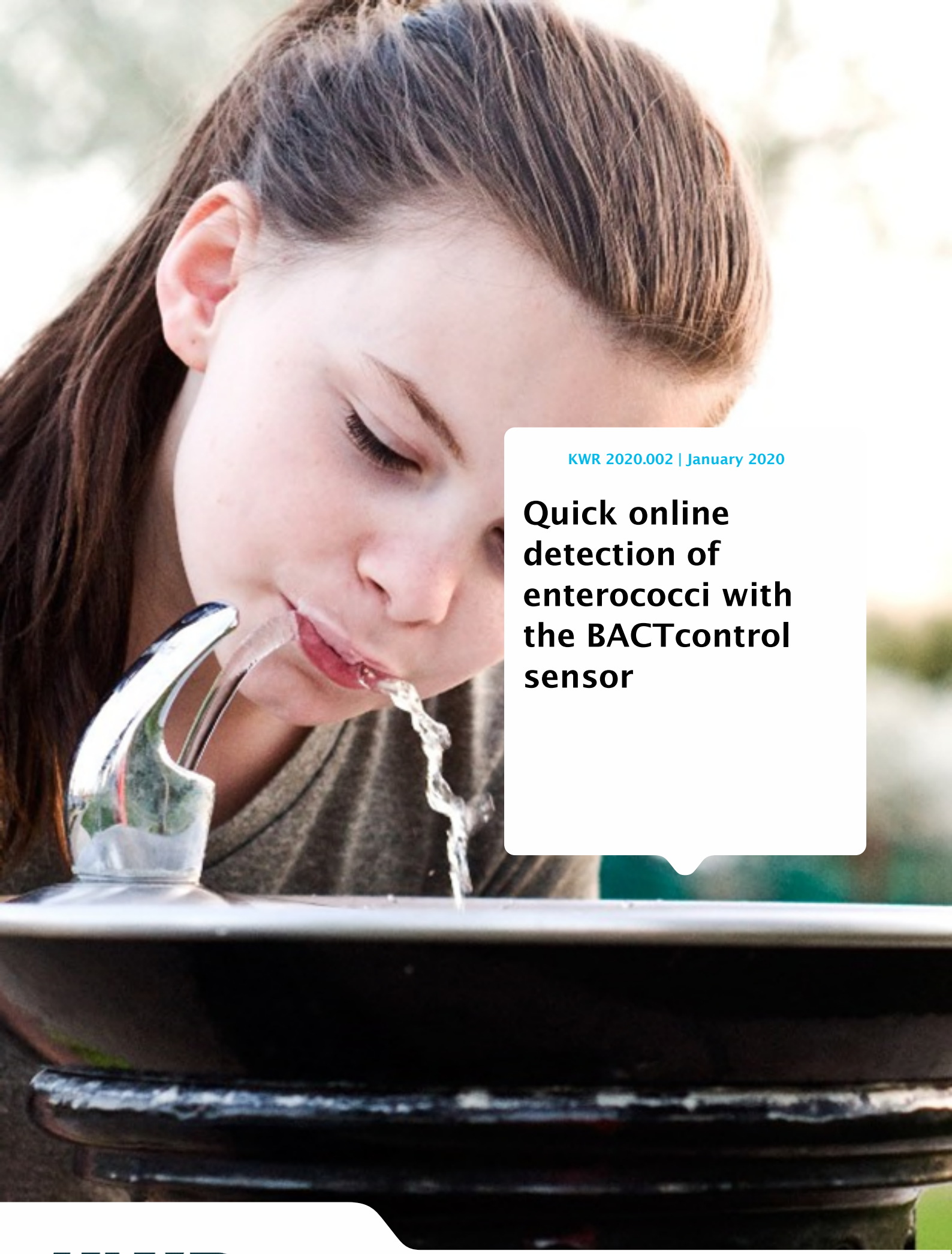

KWR 2020.002 | January 2020

Quick online detection of enterococci with the BACTcontrol sensor

KWR

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Quality Assurance
Gertjan Medema

Authors
Nikki van Bel, Gertjan Medema (KWR, all chapters), Jeroen van Rijn (KWR, chapter 2), Jaap van den Dries and Joep Appels (microLAN, chapters 1, 6), Rob Hoefnagel (Hoogheemraadschap Delfland, chapters 5.2, 5.3, 6), Eric Penders (Het Waterlaboratorium, chapters 3, 5.4, 6), Jentina Schuurman (Vitens, chapters 4, 6).

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More information
dr. Nikki van Bel
T 030-6069516
E Nikki.van.Bel@kwrwater.nl

PO Box 1072
3430 BB Nieuwegein
The Netherlands

T +31 (0)30 60 69 511
F +31 (0)30 60 61 165
E info@kwrwater.nl
I www.kwrwater.nl

Summary

The overall objective of this study was to further develop the enterococci assay on the BACTcontrol and evaluate the applicability for analysis of drinking water and surface water (bathing water, drinking water intake) in a laboratory and on-site setting.

The study first optimized the enterococci assay on the BACTcontrol (laboratory studies at KWR and HWL) to improve the detection limit and the reproducibility of the relation between the BACTcontrol signal and culturable enterococci concentrations in water samples. Subsequently, the BACTcontrol enterococci assay was applied in drinking water settings (Vitens Laboratory and HWL) and two recreational water settings (Hoogheemraadschap van Delfland). The results for the performance characteristics tested in this study are:

User experience: the users indicated that the BACTcontrol enterococci assay is easy and can be run at laboratory and semi-continuous mode, but also highlighted technical limitations that hamper semi-continuous operation and low maintenance. Several recommendations were made for improvements of the BACTcontrol system operation and maintenance based on the users' experience.

Time-to-result: a key characteristic of the BACTcontrol enterococci assay is the short time-to-result: the results of the BACTcontrol are available within 2 hours after sampling, while the culture method takes one to two days.

Background signal (noise): the background signal of the BACTcontrol when monitoring drinking water in an on-line setting is low (0 – 4 pmol/min), but can also be high. It is not clear what determines the difference in the background level between drinking water sites. Analyzing the cause of the differences could provide information about what water quality parameters impact the BACTcontrol enterococci assay signal.

Linearity: the BACTcontrol enterococci assay is linear when different volumes of the same sample are tested.

Reproducibility: in the same (bathing water) sample, the BACTcontrol has a low standard deviation (6.5 %) compared to the culture method (35 %). Between sample reproducibility is low and makes it difficult to translate the BACTcontrol activity reading to culturable enterococci concentrations and existing water quality standards and hence to communicate with process operators or others about the significance of the results. Rinsing did (not consistently) improve the BACTcontrol signal, as did cleaning of the reaction chamber and ceramic filter, suggesting that matrix effects do reduce the BACTcontrol signal. Also carry-over between subsequent assays without proper cleaning after the assay occurred. A more rigorous cleaning and rinsing protocol of the reaction chamber after each sample may increase the BACTcontrol signal more consistently.

Detection limit: previous studies showed that he dose response between culture and enzymatic activity was visible as long as the BACTcontrol sample contained >10 colony forming units (cfu) of enterococci. This study showed that the BACTcontrol enterococci assay did not show a limit-of-detection that is low enough for assaying drinking water

at a level of 1 cfu per 100 ml, even after optimization and increasing the sample volume to 1000 ml.

Correlation with reference method: despite the optimization steps, each of the users of the BACTcontrol assay found a low correlation between the BACTcontrol signal and the number of culturable enterococci between samples.

The overall outcome of this study is that successful deployment of the BACTcontrol for semi-continuous monitoring is hampered by the low between-sample reproducibility, as indicated by the two bathing site tests. The only site with a correlation between BACTcontrol signal and culturable enterococci was with the Klaarbeek at Epe, tested in offline mode in the Vitens laboratory by taking samples at the site, transport them to the lab and analyze them with the BACTcontrol and conventional culture assay. This correlation could have been impacted by the presence of a number of the same samples with different sample volumes in this data-set, but testing the BACTcontrol in semi-continuous mode at this site (provided that the technical issues are resolved), and using 1 liter volumes, could indicate whether application of the BACTcontrol enterococci assay is feasible in this surface water.

Contents

1 Introduction

1.1 Online sensoring of fecal microorganisms

Worldwide, faecal pollution is the most significant threat to health via water. The most important parameters for monitoring the (microbiological) safety of drinking and bathing water are *Escherichia coli* and intestinal enterococci. These parameters are included in all drinking and bathing water legislation. Currently, drinking and bathing water is examined for *E. coli* and intestinal enterococci with culture methods with a time-to-result of 1 or 2 days. These long waiting times don't allow rapid detection of contamination events that pose a risk to the water users or to rapidly determine the effect of control actions in response to contamination events. Hence, water utilities and authorities have the desire for rapid methods to detect faecal contamination. That need is mainly fueled by (i) the wish for rapid detection of contamination events that may occur in drinking water due to e.g. pipe breaks or maintenance and in bathing water due to combined sewer overflows, manure spills, dense bather populations etc. (ii) the disadvantages of the long waiting times, after work in the network before pipe sections can be put in service again or a beach can be re-opened and (iii) desire to rapidly assess whether contamination control measures are effective. As indicated above, the long waiting times are caused by the current microbiological culture methods. In the past years, several rapid methods have been developed for the detection of *E. coli*, such as RT-PCR or the BACTcontrol. However, their application is still limited, because *E. coli* and intestinal enterococci both need to be tested and there is no rapid method for intestinal enterococci available. Enterococci are a relevant addition since epidemiological investigations have shown a relation between the enterococci in (bathing) water and the risk of gastro-intestinal health complaints in bathers (Wade et al, 2003; Fewtrell and Kay, 2015).

1.2 General and more specific information BACTcontrol

The BACTcontrol detects microbiological activity in water samples using enzymatic reactions that make specific bacteria visible for fluorescence detection. The BACTcontrol monitors the different enzyme activities as indicators for the presence of bacterial contamination. The enzyme activity is detected by adding a substrate-specific reagent containing fluorescent indicators (see Table 1). The reagents hydrolyse with the enzymes to 4-methylumbelliferone (MUF) which fluoresces after excitation via UV irradiation (λex 360 nm; λem 450 nm).

TABLE 1. FECAL INDICATOR SPECIFIC ENZYMES DETECTED AND REAGENTS USED IN BACTCONTROL ONLINE MONITORING DEVICE

Target organism	Enzyme	Reagent
E. coli	ß-glucuronidase (GLUC)	4-methylumbelliferyl-ß-D-glucuronide (MUG)
Coliforms	ß-galactosidase (GAL)	4-methylumbelliferyl-ß-D-galactopyranoside (MUGal)
Enterococci	ß-glucosidase (GLUCAN)	4-methylumbelliferyl-ß-D-glucopyranoside (MUGlu)
Total activity	alkaline phosphatase (ALP)	4-methylumbelliferyl-ß-D-phosphate (MUP)

The BACTcontrol analyser consists of a reactor with two chambers that are separated by a ceramic reusable filter with a pore size of 0.45µm. In the reactor, the water sample is concentrated by the filter, the temperature is stabilized and the enzymatic reaction is started, while the water sample is constantly stirred by a magnetic stirrer. Further the device includes a fluorescence detector to measure the enzymatic activities (Figure 1).

Prior to each measurement, the water sample is pumped from the water source through the reactor chamber at flow rates from 1 to 24 ml per minute, the time needed for the filtering depends on the volume that has to be filtered and the condition of the filter. The sampled water volume is also measured by the pump during this process.

After setting the temperature inside the reaction chamber to the optimum temperature (44 ± 0.1 ˚C for GLUC, 36 ± 0.1 ˚C for GAL, 37 ± 0.1 ˚C for GLUCAN, 45 ± 0.1 ˚C for

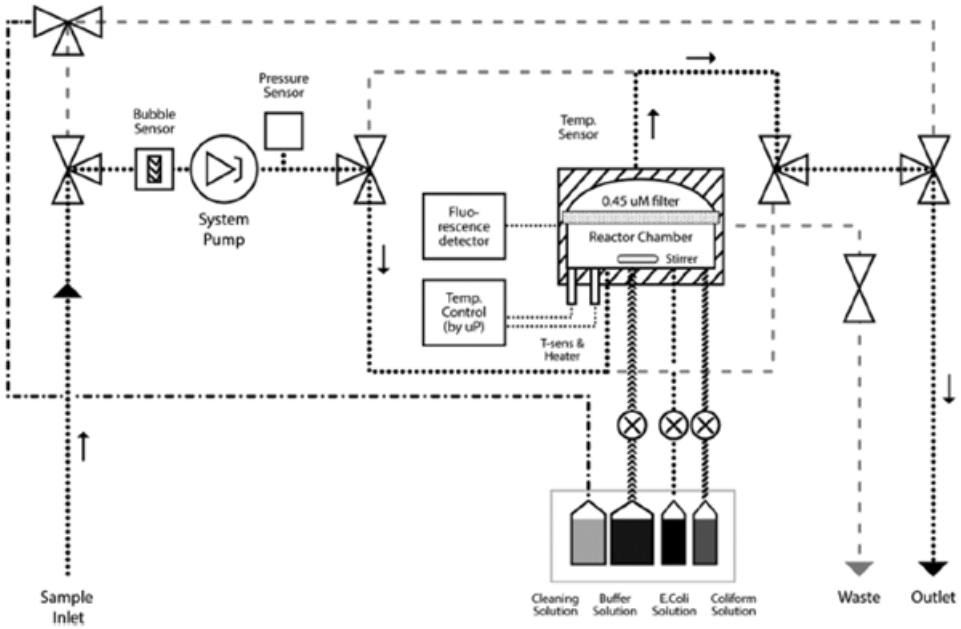

FIGURE 1. SCHEMATIC OVERVIEW OF THE BACT CONTROL SYSTEM

ALP), the stabilization followed by the actual measurement of the fluorescence intensity can take place during a 20-minute incubation period. The fluorescence intensity of the fluorometer has been calibrated using a standard with a concentration of 1,000 nM MUF. This calibration allows the fluorometer to measure the production rate of MUF, which directly corresponds to the hydrolysis rate of the substrate. The fluorescence intensity is converted into MUF production per time and volume (pmol MUF * min * 100 ml^{-1}).

The increase in fluorescence is automatically saved to the BACTcontrol computer and the slope of the signal in the steady state phase is used to calculate the enzymatic activity by ordinary least square linear regression analysis. Furthermore, the software calculates a limit of detection (DL) for each measurement performed. For this statistical approach, the measurement is regarded as significant if the average signal during the measurement exceeds threefold the standard deviation in relation to the theoretical zero line of the reaction. The DL calculation is determined after the stabilization period

(only during 20 minutes of incubation), from where the slope of the regression curve is determined until the end of this phase.

After each measurement, a cleaning / disinfection procedure is performed by the device, which comprises the injection of a chlorine solution and a heating procedure within the reactor to eliminate residues of the measuring process within the system.

1.3 Previous studies into enterococci detection with BACTcontrol

This project is a follow-up of an earlier study in which the enterococci analysis on the BACTcontrol was developed (KWR 2016.076). The BACTcontrol provides substrate to a specific enzyme and detects the product of the enzymatic activity. The ß-glucosidase enzyme is specific for enterococci and was shown to be able to convert the substrate, which is labelled with a fluorophore (4-Methylumbelliferyl-ß-D-glucopyranoside) and thereby releases the fluorophore 4-MU. The intensity of the fluorescence is a measure of the enzyme activity and can be measured by the BACTcontrol. Experiments with lab-cultured enterococci bacteria in the BACTcontrol did not show any result, which is similar to the *E. coli* analysis on the BACTcontrol and what is described in literature (George, et al., 2000). Only environmental enterococci or *E. coli* bacteria (derived from for example surface water or wastewater treatment plant (WWTP) effluent) produce a signal in the BACTcontrol. Therefore, all tests in this study were conducted with environmental enterococci.

The previous study showed a good dose-response relation between the number of environmental enterococci (as measured with the standard culture assay) and the enzymatic activity measured with the BACTcontrol within single water samples. This dose-response was not reproducible between water samples, especially for water samples derived from different water bodies. The study concluded that the water matrix has a significant impact on the BACTcontrol results.

The dose response between culture and enzymatic activity was visible as long as the BACTcontrol sample contained >10 colony forming units (cfu) of enterococci. Measurements of drinking water without enterococci showed consistently low enzymatic activity. The background enzymatic activity did differ largely (factor 10 – 100) between drinking water from different locations.

Upon finishing the earlier study several research questions and uncertainties remained:

- Only off-line laboratory experiments were performed. No experience was available on enterococci measurements in an on-line field application of the BACTcontrol.
- Enterococci can be measured with the BACTcontrol, but the relation between BACTcontrol signal and culturable enterococci varied considerably between water samples/sample locations. Therefore, it would be difficult to relate a BACTcontrol signal in a new sample/sample location to the concentration of culturable enterococci. The water matrix appeared to have a large effect on this relationship.
- The detection limit of the BACTcontrol would make it suitable for enterococci measurements in surface water or recreational waters, but the analysis is not yet sensitive enough to test for compliance testing of drinking waters with the EU/NL drinking water directive (<1 enterococci cfu/100 ml).

1.4 Study objective

The overall objective of this study was to further develop the enterococci assay on the BACTcontrol and evaluate the applicability for analysis of drinking water and surface water (bathing water, drinking water intake) in a laboratory and on-site setting.

The study consisted of multiple parts:

1. Primary optimization of enterococci assay on the BACTcontrol (laboratory studies at KWR)

This part aimed to optimize the enterococci assay on the BACTcontrol to improve the detection limit and the reproducibility of the relation between the BACTcontrol signal and culturable enterococci concentrations in water samples. The specific objective of this part was to optimise the BACTcontrol enterococci assay by:

- testing the effect of different lysis buffers, buffer strengths, sample volumes and sonification and reaction times on the relation between culturable enterococci and the BACTcontrol signal;
- testing the effect of rinsing of the BACTcontrol reaction chamber with buffer to reduce matrix effects.

2. Secondary optimization of the enterococci assay on the BACTcontrol (Laboratory studies at Het Waterlaboratorium)

This part aimed to further optimize the BACTcontrol enterococci assay, with as specific objectives:

- What is the influence of an even lower concentration of substrate (12.5 mM) on the measurement of enterococci with the BACTcontrol?
- Can differences in results between different (surface) water types, in relation to the number of culturable enterococci, be prevented by using an extra rinsing step in the protocol?
- Can the detection limit be lowered by filtering and analyzing a larger sample volume?
- What is the normal background signal of the BACTcontrol when tap water is measured?

3. Application of the BACTcontrol enterococci assay in a drinking water setting (Vitens Laboratory)

This activity was to test the application of the BACTcontrol enterococci assay in a drinking water setting. The specific objectives were to:

- Expand the comparison of BACTcontrol vs culturable enterococci assay to other surface waters (production site Epe).
- Compare the BACTcontrol and culture enterococci assay in drinking water samples.
- Collate user experience with the BACTcontrol in a water laboratory environment.

4. Application of the BACTcontrol enterococci assay at field sites (Hoogheemraadschap van Delfland and Het Waterlaboratorium)

This activity aimed to test the performance of the BACTcontrol for the enterococci assay in the field, at two bathing sites and one surface water intake point for drinking water supply.
The specific objectives were to:

- Collate user experience with the BACTcontrol in the field for on-site, semi-continuous measurement of enterococci.
- Compare the BACTcontrol and culture enterococci assay in field settings.

2 Primary optimization of the BACTcontrol enterococci assay (KWR)

2.1 Introduction

The previous study showed that the detection limit of the BACTcontrol enterococci assay is too high for application for regulatory compliance monitoring of drinking water. The BACTcontrol assay conditions were developed for detection of ß-galactosidase and ß-glucuronidase activity in coliforms and *Escherichia coli* and the same conditions have been applied to develop the ß-glucosidase assay for enterococci. Since enterococci are Gram-positive bacteria, they may be more resistant to the surfactants used in the BACTcontrol assay to permeabilise the bacterial membrane. More rigorous procedures might be needed to improve the access of the substrate to the ß-glucosidase enzyme in enterococci. In addition, the previous study showed a high within sample, but low between sample reproducibility of the BACTcontrol enterococci assay, when the BACTcontrol results were compared to enterococci counts on culture media, using the conventional method. For deployment of the BACTcontrol enterococci assay in water testing, a consistent relation between BACTcontrol signal and enterococci counts on culture media is important to be able to interpret the BACTcontrol assay. The difference between samples may have been caused by components in the sample matrix that affect the substrate availability, enzyme reaction or fluorescence produced. After filtration of the sample in the BACTcontrol, the reaction chamber of the BACTcontrol still contained a small volume of sample. To reduce matrix effects and improve between-sample reproducibility, it could be beneficial to replace this sample volume in the BACTcontrol reaction chamber by reaction buffer.

2.2 Objective

The specific objective of this part was to optimize the enterococci assay on the BACTcontrol to improve the detection limit and the reproducibility of the relation between the BACTcontrol signal and culturable enterococci concentrations in water samples.

- testing the effect of different lysis buffers, buffer strengths, sample volumes and sonification and reaction times on the relation between culturable enterococci and the BACTcontrol signal;
- testing the effect of substrate concentration on the BACTcontrol signal;
- testing the effect of rinsing of the BACTcontrol reaction chamber with buffer to reduce matrix effects.

2.3 Methods

2.3.1 Enterococci culture method

The number of enterococci in the water samples was determined according to NEN-EN-ISO 7899-2. In short the water sample was filtered through a membrane filter and the filter was incubated on Slanetz and Bartley agar plates for 44 ± 4 hours at 36 ± 2˚C. The number of colonies was counted and the concentration in cfu/100 ml calculated.

The number of enterococci was also determined using the Enterolert-QuantiTray2000 (IDEXX), for this water samples were measured undiluted and/or 1:10 diluted in sterile drinking water. After incubation the number of positive wells was used to calculate the MPN/100 ml. In most cases only the MPN/100 ml, excluding the confidence interval, was given.

2.3.2 BACTcontrol measurement

2.3.2.1 Measurement conditions

The conditions of the BACTcontrol were:

- 10 minutes calibration time (20 mins used in chapter 3, 4 and 5)
- 60 minutes measurement time (40 mins used in chapter 3, 4 and 5)
- Volume reaction chamber: 10 ml (in the newer version 2ml, used in chapter 5.1-5.3)
- Substrate volume: 250 µl
- Substrate concentration: 12.5 – 100 mM
- Buffer volume: 250 µl
- Filtration volume: 100 – 1000 ml

In chapter 2, the older version of the BACTcontrol was used, while in chapters 3, 4 and 5 the newer model, optimized in-house at MicroLAN on the basis of the *E. coli* signal, was employed.

2.3.2.2 Substrate 4-Methylumbelliferyl-ß-D-glucopyranoside

4-Methylumbelliferyl-ß-D-glucopyranoside (4-MU-ß-D-glucopyranoside) was used as substrate. The compound contains a fluorophore (4-methylumbelliferyl [4-MU], red in Figure 2) and the substrate for the enzyme ß-glucosidase (ß-D-glucopyranoside, blue in Figure 2) which is specific for enterococci. Upon cleavage of the bond between 4-MU and ß-D-glucopyranoside, 4-MU is free and fluoresces with light emission at 448 nm upon excitation by light of 365 nm.

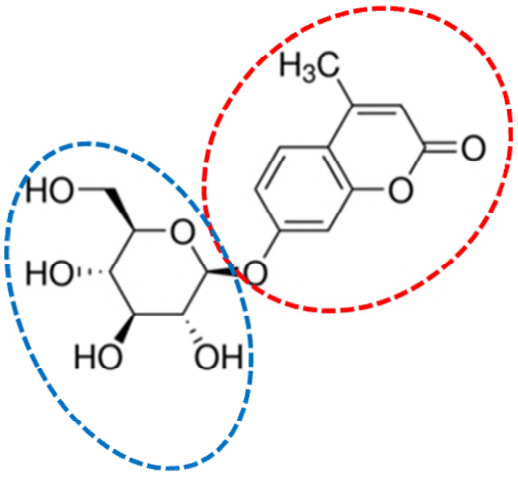

FIGURE 2. STRUCTURAL FORMULA OF 4-METHYLUMBELLIFERYL-ß-D-GLUCOPYRANOSIDE. RED: 4-METHYLUMBELLIFERYL. BLUE: ß-GLUCOPYRANOSIDE.

2.3.2.3 Gly-Gly buffer

Several buffers were tested, of which most are based on a buffer containing Gly-Gly as buffering component. The buffer described below is called the ‘normal’ buffer in this report.

For the 'normal' buffer the following compounds were mixed:

- 10 ml 1M GlyGly, pH8.8 (Sigma-Aldrich, G1002)
- 0,5 ml/100 ml Triton-X-100 (Sigma-Aldrich, 93443)
- 1,57g/100 ml Sodium thiosulfate Pentahydrate (Sigma-Aldrich, 217247)
- 0,1% (w/v) Sodiumazide, added as dry powder (Sigma-Aldrich, 438456)
- Add ultrapure water to 25 ml

2.3.3 Comparison of substrate suppliers

The costs of the substrate 4-MU-ß-D-glucopyranoside at Sigma-Aldrich are high. Therefore another supplier of the substrate was found and the substrates were compared to each other. 4-MU-ß-D-glucopyranoside was obtained from Sigma-Aldrich (M3633) and Alfa Aeser (J66630). We compared the degradation rate and the BACTcontrol signal of enterococci in water samples using the substrate from both suppliers.

2.3.3.1 Degradation rate of ß-glucopyranoside substrate

4-MU-ß-D-glucopyranoside was dissolved in DMSO. A dilution series (12.5 mM, 25 mM, 50 mM and 100 mM) was made in DMSO and stored at two temperatures (4 and 20˚C) in the dark in triplicate. After 0, 3, 6, 9, 12 and 15 weeks the amount of 4-MU formed was measured using a spectrophotometer. A calibration curve of 4-MU (Sigma-Aldrich, M1381) was included in the measurements to calculate the concentration of 4-MU that was formed.

2.3.3.2 Enzymatic activity of enterococci in drinking and surface water

Two types of Dutch drinking water (KWR, produced from ground water; Weesperkarspel, produced from surface water) and Dutch surface water (Waal at Vuren, Lekkanaal at Nieuwegein) were sampled and stored at 4˚C. One hour before the start of the BACTcontrol measurement the water sample was heated up in a water bath at 20˚C.Of each water sample 100 ml and 1000 ml were sequentially measured in the BACTcontrol with both substrates, according to the schema in Table 2. The number of enterococci was also determined with plate count and with the Enterolert method. For these measurements Gly-Gly buffer and 100 mM substrate were used.

TABLE 2. EXAMPLE OF EXPERIMENTAL SCHEME IN MEASURING ONE WATER SAMPLE UNDER DIFFERENT CONDITIONS. SUBSTRATE CONCENTRATION: 100 MM, NORMAL BUFFER, MEASUREMENT TIME: 60 MINUTES.

Day	Substrate supplier	Analysis	Filtration volume	Water type	Enterolert and culture
1	Sigma	Blank	100 ml	Drinking water	
1	Sigma	Measurement	100 ml	Surface/drinking water A	
1	Sigma	Measurement	1000 ml	Surface/drinking water A	2x
1	Aeser	Blank	100 ml	Drinking water	
1	Aeser	Measurement	100 ml	Surface/drinking water A	
1	Aeser	Measurement	1000 ml	Surface/drinking water A	2x
2	Aeser	Blank	100 ml	Drinking water	
2	Aeser	Measurement	100 ml	Surface/drinking water B	
2	Aeser	Measurement	1000 ml	Surface/drinking water B	2x
2	Sigma	Blank	100 ml	Drinking water	
2	Sigma	Measurement	100 ml	Surface/drinking water B	

2	Sigma	Measurement	1000 ml	Surface/drinking water B	2x

2.3.4 Comparison of buffers

The effect of several buffers on enterococci was tested in two ways. To allow for the enzymatic cleavage of 4-MU-ß-D-glucopyranoside by ß-glucosidase that is present in the enterococci, the buffer should permeabilise or break down the cell wall of the enterococci to release the enzyme. Due to the permeabilised or degraded cell wall the enterococci are assumed to be no longer culturable. This assumption was used to test the effect of different concentrations of Triton X100 as surfactant and lysozyme to the normal buffer, or use sonication, or use different lysis buffers on environmental enterococci by incubating effluent (3, 10 or 30 ml) from a wastewater treatment plant (Kaatsheuvel) containing environmental enterococci with several buffers in a range of conditions (bacteria in suspension or on a filter; incubation at 20˚C [RT: room temperature] or at 37˚C). The number of culturable enterococci in after buffer treatment was measured using the standard culture method. After filtration of the sample the membrane was flushed with sterile drinking water to remove the buffer and stop the reaction.

A buffer that can permeabilise or break down the cell wall is not necessarily compatible with the BACTcontrol system and the enzymatic process that needs to take place to degrade substrate in the reaction chamber. Therefore, often the incubated samples were also measured in the BACTcontrol to test the effect of the buffer on enzymatic activity. The following buffers were tested:

- Gly-Gly buffer:
  - Normal (0.5% Triton X-100, chapter 2.3.2.3)
  - 0% Triton X-100
  - 5% Triton X-100
  - 10% Triton X-100
  - Normal + 1.0 mg/ml lysozyme
  - Normal + 0.1 mg/ml lysozyme
  - Normal + 0.02 mg/ml lysozyme
  - Normal + 20% IGEPAL CA-630
- Sonification: 2, 4 or 8 minutes at 45%
- BacTiter-Glo Buffer (Promega, G8232), according to protocol
- Fastbreak cell lysis reagent (Promega, V8571), according to protocol
- Passive Lysis Buffer (Promega, E1941), according to protocol

2.3.5 Effect of rinsing

The effect of rinsing the reaction chamber of the BACTcontrol with ultrapure water after filtration of the water sample on the reproducibility of the measured enzymatic activity was tested according to the scheme in Table 3. Surface water was sampled at the Lekkanaal near Nieuwegein or at the Waal near Vuren and stored at 4˚C. One hour before the start of the BACTcontrol measurement the water sample was heated up in a water bath at 20˚C.

TABLE 3. EXPERIMENTAL SCHEME FOR TESTING THE EFFECT OF RINSING BY MEASURING A SURFACE WATER SAMPLE (LEKKANAAL) WITH AND WITHOUT RINSING WITH ULTRAPURE WATER AT DIFFERENT SUBSTRATE CONCENTRATIONS. SUBSTRATE SUPPLIER: SIGMA-ALDRICH.

	Substrate (mM)	Rinsing	Analysis	Volume (ml)	Water type	Enterolert and culture
1	100	No	Blank	100	Drinking water	
2	100	Yes	Measurement	1000	Lekkanaal	2x
3	100	No	Measurement	1000	Lekkanaal	
4	25	No	Blank	1000	Drinking water	
5	25	Yes	Measurement	100	Lekkanaal	2x
6	25	No	Measurement	100	Lekkanaal	

2.4 Results

2.4.1 Comparison of 4-MU-ß-D-glucopyranoside substrate supplier and concentration

2.4.1.1 Degradation of 4-MU-ß-D-glucopyranoside substrate

During storage of a substrate batch, autolysis may occur, liberating the fluorophore from the substrate. This implies that the background fluorescence of the substrate increases. Using a dilution range of the substrate that was stored at 4˚C or 20˚C for 16 weeks, it was tested if and how fast 4-MU-ß-D-glucopyranoside breaks down into ß-D-glucopyranoside and the fluorescent group 4-MU.

During the testing period of 16 weeks very little 4-MU was formed during storage at 4˚C or 20˚C (Figure 3). There was no difference in storage temperature and substrate supplier.

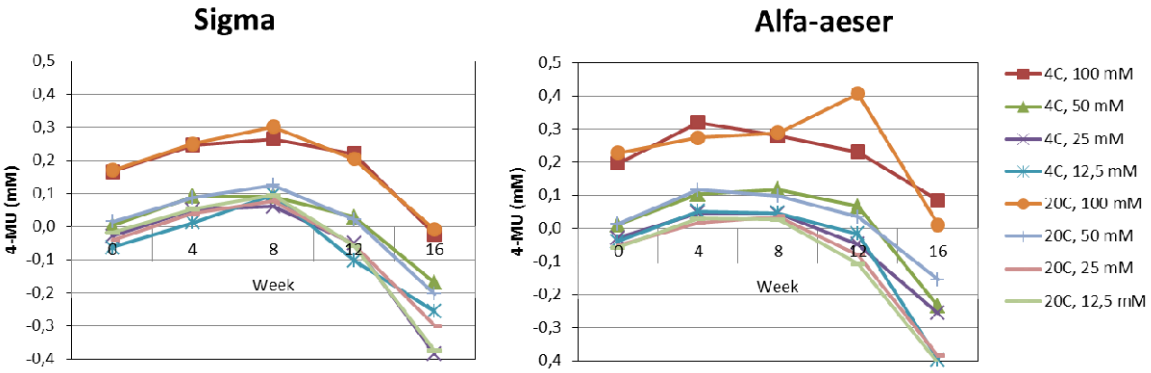

FIGURE 3. DEGRADATION OF THE SUBSTRATE 4-MU-ß-D-GLUCOPYRANOSIDE BY MEASURING THE FORMATION OF 4-MU DURING 16 WEEKS STORAGE.

2.4.1.2 Enzymatic activity of drinking and surface water

In addition to testing the degradation rate of the two substrates, also their behaviour in the BACTcontrol was tested. In drinking water samples, there was no difference in enzymatic activity from both substrates when measuring 100 ml or 1000 ml (Table 4).

In surface water samples, the Aeser-substrate yielded much lower enzymatic activities compared to the Sigma-substrate. This was more prominent when the larger volume, and thus larger number of enterococci, was assayed. The Sigma substrate showed a dose response, while the Aeser substrae did not.

In addition, we observed, as in the previous project, that the water matrix affected the enzymatic activity signal of the BACTcontrol. In the water sample from the Lekkanaal, measured with Sigma-substrate, 44.5 cfu enterococci gave an enzymatic activity of 186.72 pmol. In comparison, 36.5 cfu enterococci from the Waal water sample yielded an enzymatic activity that is only 63.25 pmol.

An additional observation was that the membrane filtration method yielded higher enterococci concentrations than the MPN methods in both surface water samples and volumes.

TABLE 4. COMPARISON OF TWO SUBSTRATE SUPPLIERS IN THE BACTCONTROL WITH TESTING DRINKING WATER AND SURFACE WATER FROM TWO LOCATIONS. SUBSTRATE CONCENTRATION: 100 MM, MEASUREMENT TIME: 60 MINUTES.

	BACTcontrol (volume tested) ml	Enzymatic activity BACTcontrol pmol/volume	Enterococci	
			cfu/volume	MPN/volume
Drinking water, 1				
Sigma	100	5.34	0	0
Sigma	1000	15.64	0	0
Aeser	100	5.63	0	0
Aeser	1000	17.72	0	0
Drinking water, 2				
Sigma	100	7.75	0	0
Sigma	1000	18.59	0	0
Aeser	100	6.13	0	0
Aeser	1000	17.28	0	0
Lekkanaal, surface water				
Sigma	100	186.72	45	25
Sigma	1000	1132.55	445	251
Aeser	100	103.15	34	22
Aeser	1000	158.58	340	224
Waal, surface water				
Sigma	100	63.25	37	19
Sigma	1000	769.68	365	195
Aeser	100	89.04	29	7
Aeser	1000	53.62	290	70

In conclusion, the Sigma-substrate performed better than the Aeser-substrate:

- While enzyme activity for drinking water was similar between both substrate suppliers; the Sigma-substrate showed higher activities and a better dose-response relation in surface waters at higher (>100) numbers of culturable enterococci.

- At lower enterococci numbers there was little difference between both substrates. However, the measured signal seemed not to exceed the background noise.
- As Sigma performed better at higher enterococci numbers, while being influenced by the water matrix, it was assumed that Sigma will also perform better when lower numbers of enterococci are measured or if the water matrix effect is smaller.
- Therefore, the Sigma-substrate was selected for subsequent experiments.

2.4.2 Effect of rinsing and varying substrate concentrations

The effect of rinsing the reaction chamber with ultrapure water after filtration of the water sample increased the enzymatic activity with a factor of about 1.5x in each of the three surface water samples and at both substrate concentrations (Table 5). In addition, the enzymatic activity of all water samples (containing 8-22 enterococci) was much higher compared to drinking water without enterococci.

A substrate concentration of 100 mM yielded 7 - 56% higher enzyme activities than 25 mM. At both concentrations the enzymatic activity in the presence of enterococci was much higher compared to drinking water.

TABLE 5. THE EFFECT OF RINSING THE REACTION CHAMBER WITH ULTRAPURE WATER AFTER SAMPLE FILTRATION IN THE BACTCONTROL USING TWO SUBSTRATE CONCENTRATIONS (25 MM AND 100 MM). MEASUREMENT TIME: 60 MINUTES. FILTRATION VOLUME: 100 ML, SUBSTRATE: SIGMA.

		Enzymatic activity BACTcontrol	Enterococci	
	Rinsing	pmol	cfu/volume	MPN/volume
Lekkanaal, surface water, 1				
100 mM	Yes	625	10.6	6.3
100 mM	No	406		
25 mM	Yes	440	8	4.1
25 mM	No	272		
Lekkanaal, surface water, 2				
100 mM	Yes	633	11.5	8.6
100 mM	No	484		
25 mM	Yes	589	14	7.4
25 mM	No	360		
Waal, surface water				
100 mM	Yes	413	22	12.9
100 mM	No	291		
25 mM	Yes	352	17	10.2
25 mM	No	186		

These experiments showed again that the water matrix influenced the enzymatic activity. In the water sample from the Waal more culturable enterococci were present, but the enzymatic activity was lower than the Lekkanaal samples with less culturable enterococci. This indicated that either the water from the Waal contained compounds that inhibit the development of the BACTcontrol signal or that the water from the Lekkanaal contains compounds that stimulated access of the substrate to the enzymes, enzymatic activity or chemical cleavage of the substrate to release the fluorophore. Rinsing of the reaction chamber may have removed (part of) these inhibiting or stimulating compounds.

We observed again that the membrane filtration yielded higher counts than the enterolert MPN method (Table 5).

In conclusion:

- Surface water may contain compounds inhibiting enzymatic activity, or compounds that (chemically) cleave the substrate thereby releasing the fluorogenic part.
- Rinsing of the reaction chamber with ultrapure water before the enzyme reaction improved the BACTcontrol signal with a factor of approx.1.5.
- The difference in enzymatic activity of 25 mM or 100 mM substrate on measuring surface water is 7-56%.
- At 25 mM the enzymatic activity level is higher than drinking water and thus this concentration seems sufficient.

The Sigma-substrate at 25 mM was chosen as adequate and save costs, and used for all upcoming experiments. If possible, the filtration chamber should be flushed with demineralized or ultrapure water after filtration of the water sample.

2.4.3 Comparison of buffers

A large set of buffers was tested in order to try to improve the results with the BACTcontrol and reduce the disturbance caused by the water matrix on the measured enzymatic activity. The effect of the buffers on the enterococci was tested in the BACTcontrol and on culture plates, as described in chapter 2.3.4.
When comparing the effect of the buffers on culturability and BACTcontrol signal, it is important to keep in mind that there may be several reasons due to which enterococci will not form colonies on culture plates, and this does not always coincide with the (predicted) release of the enzyme ß-glucosidase from the cell into the environment (Table 6).

TABLE 6. POSSIBLE EFFECTS OF THE LYSIS BUFFER ON THE CULTURABILITY OF ENTEROCOCCI AND RELEASE OF THE ENZYME □-GLUCOSIDASE.

Effect of buffer on enterococci	Culturable	Predicted release of enzyme
Complete lysis	No	Yes
Permeabilisation	No	Perhaps
Damaged	No	Perhaps
Dead, but cell membrane/wall remains intact	No	No
No effect	Yes	No
Resuscitation	Yes	No

2.4.3.1 Promega lysis buffers

Three lysis buffers of Promega were tested: Fastbreak, BacTiterGlo and Passive Lysis Buffer. According to Promega's protocol, these buffers are designed to completely lyse bacterial cells and thus release all contents, containing enzymes. However, these buffers are not designed to maintain the structure of the enzyme and thus may degrade or change the structure due to which enzymes become inactive.

Upon incubation with any of the Promega buffers, nearly all enterococci present in WWTP effluent became unculturable (Figure 4). Incubation temperature of 20 or 37°C, volume of effluent used and whether the test was done in suspension or on a filter did not influence the inactivation.

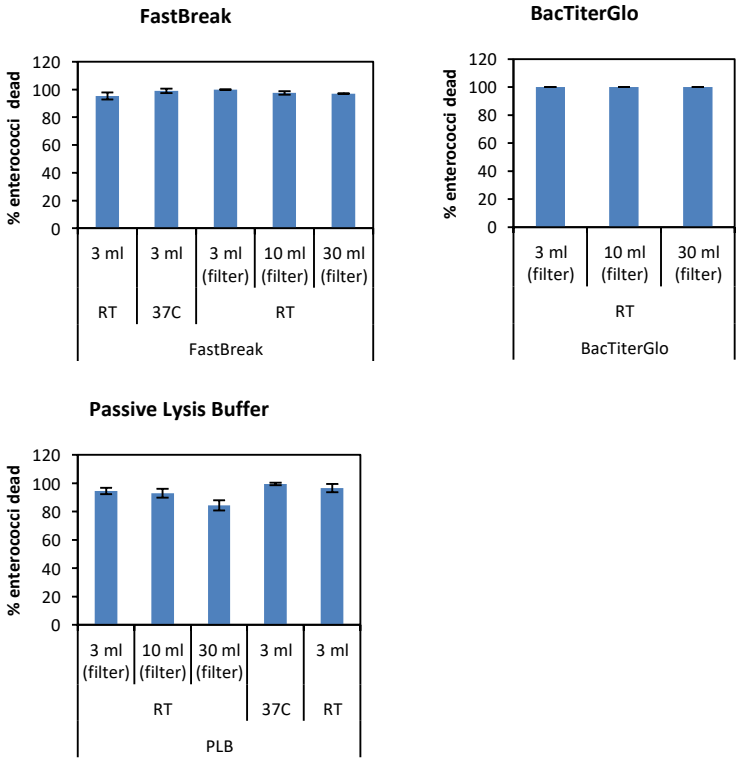

FIGURE 4. PERCENTAGE (%) ENTEROCOCCI BACTERIA UNCULTURABLE AFTER TREATMENT WITH THE FASTBREAK-, BAC TITER GLO AND PASSIVE LYSIS BUFFER OF PROMEGA. SHOWN IS AVERAGE (N= 2-3) WITH STANDARD DEVIATION. TEST WATER: WWTP EFFLUENT

However, these buffers are not very practical in the BACTcontrol-setting: the BacTiterGlo buffer has to be applied to bacteria on a filter and not in suspension, the Passive Lysis Buffer has to be diluted 1:5 and the FastBreak buffer requires a 1:10 dilution. As a consequence, relatively large amounts of buffer should be added in the BACTcontrol-system: 2 ml Passive Lysis Buffer or 1 ml FastBreak buffer.

Using WWTP effluent, the FastBreak buffer was tested in the BACTcontrol-system alongside with the normal buffer (Figure 5). This showed that, despite comparable numbers of enterococci in the water of the 10-ml tests, the enzymatic activity was much

lower when using the FastBreak buffer compared to when the normal buffer was used. This was apparent in both tests.

Although the FastBreak buffer rendered nearly 100% of the enterococci unculturable, the enzymatic reaction to release the fluorescent group from the substrate was strongly inhibited. This shows that there is a delicate balance that should be achieved by the buffer: lyse or permeabilise nearly all enterococci while keeping the enzymes intact and without inhibiting the substrate conversion.

FIGURE 5. COMPARISON OF FASTBREAK-BUFFER WITH THE NORMAL BUFFER IN THE BACTCONTROL. TEST WATER: WWTP EFFLUENT, MEASUREMENT TIME: 60 MINUTES SUBSTRATE: SIGMA.

	BACTcontrol (volume tested) ml	Enzymatic activity BACTcontrol pmol	Enterococci	
			cfu/vol	MPN/vol
Test 1				
Normal buffer	10	66.2	175	169
FastBreak buffer	10	20.4	190	122
Normal buffer	42	123.7	651	489
Test 2				
Normal buffer	10	53.1	115	114
FastBreak buffer	10	18.6	115	57
Normal buffer	10	44.2	47	40

2.4.3.2 Normal buffer with lysozyme

Lysozyme is an enzyme often used to lyse bacterial cells. However, lysozyme has to be stored frozen and should be used directly after thawing. Lysozyme buffers are therefore not directly applicable for online measurements (for which the buffer should be stable at room temperature for several months), but was used in this study as a research tool.

Incubation of WWTP effluent with the normal buffer and added lysozyme killed enterococci, with the efficiency depending on the lysozyme concentration, the volume of WWTP effluent that was used and whether the test was performed in suspension or on a filter (Figure 6). Lower lysozyme-concentrations lead to lower die-off percentages. Higher test volumes (more bacteria and interfering particles present) had the same effect and rendered the lysis buffer less efficient. The lysis buffer was more effective when incubated with the bacteria in suspension compared to incubation on a filter, probably because the lysis buffer had better access to all bacteria when in suspension compared to when bacteria concentrated on the filter.

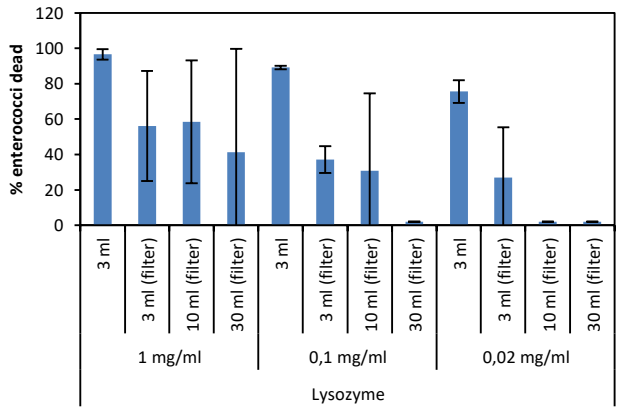

FIGURE 6. PERCENTAGE (%) ENTEROCOCCI BACTERIA UNCULTURABLE BY ADDITION OF DIFFERENT CONCENTRATIONS LYSOZYME TO THE NORMAL BUFFER. SHOWN IS AVERAGE (N = 2) WITH STANDARD DEVIATION. TEST WATER: WWTP EFFLUENT

The buffer containing lysozyme was also tested in the BACTcontrol with WWTP effluent with high concentrations of culturable enterococci (Table 7). The presence of lysozyme reduced the BACTcontrol signal considerably. So, incubation of the lysozyme buffer with WWTP effluent rendered a large percentage of enterococci unculturable and probably lysed, but did not improve (but reduced) the BACTcontrol signal.

TABLE 7. COMPARISON OF THE NORMAL BUFFER WITH LYSOZYME IN THE BACTCONTROL. TEST WATER: WWTP EFFLUENT, MEASUREMENT TIME: 60 MINUTES, SUBSTRATE: SIGMA.

	BACTcontrol (volume tested) ml	Enzymatic activity BACTcontrol pmol	Enterococci		Activity/ enterococ pmol/cfu
			cfu/vol	MPN/vol	
Test 1					
Normal + 0 mg/ml lysozyme	10	50,1	120	155	0.42
Normal + 0.01 mg/ml lysozyme	10	33,0	125	91	0.26
Normal + 0.1 mg/ml lysozyme	10	32,0	145	102	0.22
Normal + 1 mg/ml lysozyme	100	224,9	1250	799	0.18
Test 2					
Normal + 0 mg/ml lysozyme	10	55,6	145	148	0.38
Normal + 0.01 mg/ml lysozyme	10	40,1	120	164	0.33
Normal + 0.1 mg/ml lysozyme	10	45,2	135	113	0.33
Normal + 1 mg/ml lysozyme	100	3,8	145	91	0.03

2.4.3.3 NP40 / IGEPAL

The nonionic detergent NP40 (or IGEPAL) is one of the most widely used in lysis buffers. It yields a milder lysis buffer compared to, for example the Promega or lysozyme buffers and is generally used when the protein functions in the bacterial cell have to be retained as best as possible.

When applied to WWTP effluent, the normal buffer without NP40 yielded only about 10-15% of the enterococci unculturable, whereas addition of NP40 led to an increased die off up until about 50% (Figure 7). A longer incubation time increased the die off by treatment with the normal buffer both with and without NP40.

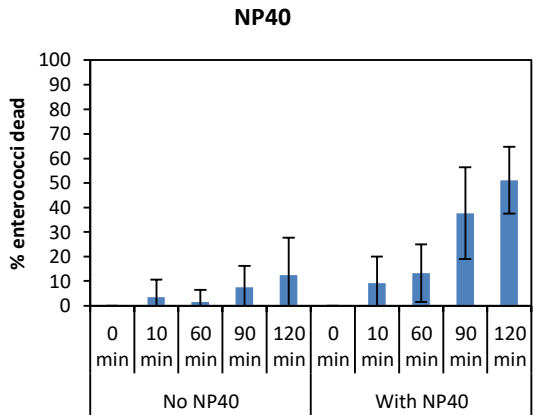

FIGURE 7. PERCENTAGE (%) OF DEAD (OR UNCULTURABLE) ENTEROCOCCI BACTERIA BY ADDITION OF NP40 TO THE NORMAL BUFFER. SHOWN IS AVERAGE (N = 4) WITH STANDARD DEVIATION. TEST WATER: WWTP EFFLUENT

In the BACTcontrol the buffer with NP40 did not yield a clear improvement in the signal compared to the buffer without NP40 (Table 8). Although in the first test the enzymatic activity with NP40 was higher (25.4 vs 19.3 pmol, also more culturable enterococci were present (225 vs 190). The calculated activity per culturable enterococci showed no difference between the two buffers. Although more enterococci die off during incubation this did not result in a higher BACTcontrol signal, suggesting that the BACTcontrol signal was slightly inhibited. Longer incubation times also led to lower enzymatic activities, both in the presence or absence of NP40.

TABLE 8. COMPARISON OF THE NORMAL BUFFER WITH AND WITHOUT NP40 IN THE BACTCONTROL. TEST WATER: WWTP EFFLUENT, SUBSTRATE: SIGMA.

	BACTcontrol (volume tested) ml	Measurement time min	Enzymatic activity BACTcontrol Pmol	Enterococci culture cfu/vol	Enterococci culture MPN/vol	Activity/ enterococ pmol/cfu
Test 1						
Normal	10	60	19.3	190	242	0.10
Normal	10	120	14.2	235	155	0.06
Normal + NP40	10	60	25.4	225	>242	0.11
Normal + NP40	10	120	16.3	265	242	0.06
Test 2						
Normal	10	60	16.6	155	199	0.11
Normal	10	120	14.3	205	143	0.07
Normal + NP40	10	60	24.3	165	245	0.15
Normal + NP40	10	120	12.9	175	246	0.07

2.4.3.4 Normal buffer: incubation time and Triton X-100 concentrations

The lysing component of the normal buffer is Triton X-100. The effect of increasing the Triton X-100 concentration and varying the incubation time was tested (Figure 8). Without Triton X-100 (0%) no enterococci became unculturable, whereas addition of 0.5%, 5% or 10% of Triton X-100 did lead to unculturable enterococci. The concentration Triton X-100 seemed less important for enterococci lysis, but due to the sometimes large variation this was difficult to determine.

A longer incubation time seemed to lead to more unculturable enterococci. Not all enterococci were inactivated by the Triton X-100: after 60 min with 0.5% Triton X-100 34% of the enterococci had become unculturable, whereas at 120 min at the same Triton X-100 concentration this was 61%. Higher Triton concentrations did not improve this percentage.

As described earlier, it is possible that the bacteria are permeabilised during incubation with the buffer, and in the BACTcontrol setting the enzymes would become available to react with the substrate. This may not be fully visible in the inactivation of culturable enterococci as during culture on agar plates enterococci may resuscitate, masking the effect of the buffer. In the BACTcontrol system, measurement times are short and nutrients are absent so resuscitation of enterococci will not occur. Therefore, the impact of Triton concentration and incubation time was tested in a large set of experiments with environmental enterococci from WWTP effluent in the BACTcontrol (Table 9).

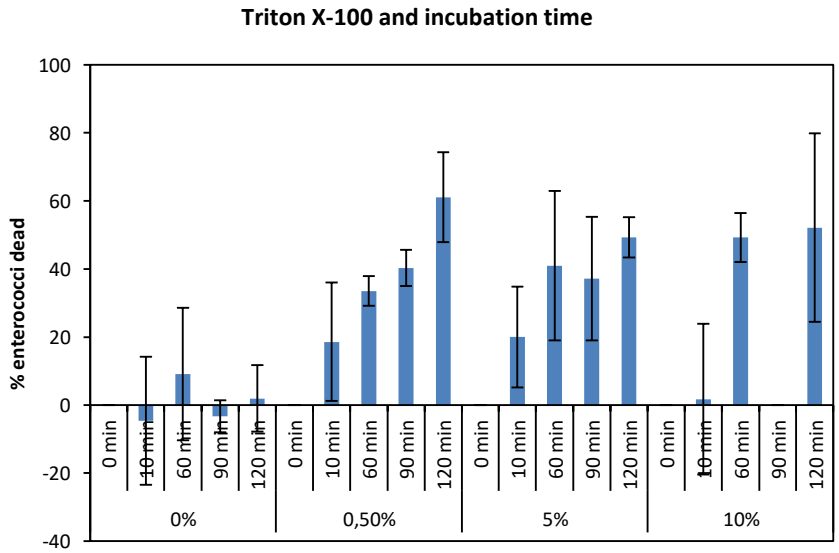

FIGURE 8. PERCENTAGE (%) OF DEAD (OR UNCULTURABLE) ENTEROCOCCI BACTERIA BY INCREASING THE INCUBATION TIME OF THE NORMAL BUFFER CONTAINING DIFFERENT TRITON X-100 CONCENTRATIONS. SHOWN IS AVERAGE (N = 2-4) WITH STANDARD DEVIATION. TEST WATER: WWTP EFFLUENT.

TABLE 9. COMPARISON OF THE NORMAL BUFFER WITH VARYING TRITON X-100 CONCENTRATIONS IN THE BACTCONTROL. TEST WATER: WWTP EFFLUENT, SUBSTRATE: SIGMA.

Triton X-100	BACTcontrol (volume tested)	Measurement time	Enzymatic activity BACTcontrol	Enterococci culture		Activity/ enterococ
%	ml	min	pmol	cfu/vol	MPN/vol	pmol/cfu
Test 1						
0.5	10 ml	60	66.2	175	169	0.38
0.5	42 ml	60	123.7	651	489	0.19
5	10 ml	60	57.0	110	122	0.52
10	10 ml	60	47.9	145	130	0.33
Test 2						
0.5	10 ml	60	53.1	115	114	0.46
0.5	10 ml	60	44.2	47	40	0.94
5	10 ml	60	52.2	130	89	0.40
10	10 ml	60	43.1	105	79	0.41
Test 3						
0.5	10 ml	60	505.5	220	233	2.30
0.5	10 ml	120	161.0	260	209	0.62
0.5	10 ml	120	145.5	155	165	0.94
5	10 ml	60	138	94	106	1.47
5	10 ml	120	1040	110	72	9.45
5	10 ml	120	382.5	150	90	2.55
Test 4						
0.5	10 ml	60	77.8	44	168	1.77
0.5	10 ml	120	69.1	47	134	1.47
0.5	10 ml	60	69.3	110	142	0.63
0.5	10 ml	120	76.6	120	146	0.64
5	100 ml	60	1217	4500	11848	0.27
5	10 ml	120	88.6	455	1595	0.19
5	10 ml	60	175.7	385	797	0.46
5	10 ml	120	9.8	395	365	0.02
Test 5						
0.5	10 ml	60	145.3	555	1203	0.26
0.5	10 ml (1:10 diluted)	120	20.9	6	8	3.48
0.5	10 ml	120	125.2	600	752	0.21
0.5	100 ml	120	858.1	5000	6820	0.17
5	10 ml (1:10 diluted)	120	18.2	4	4	4.55
5	100 ml	120	790.2	2600	2782	0.30
Test 6						
5	10 ml	120	100.5	295	424	0.34
5	10 ml	60	150.4	220	580	0.68

Test 1 and 2 showed varying results when using the 0.5%, 5% and 10% buffers. In test 1 the 5% buffers seemed to yield a higher BACTcontrol signal per enterococci compared to the 0.5% and 10% buffer. However, in test 2 the BACTcontrol activity with 5% Triton was not higher. The 10% Triton X-100 buffer did not perform better compared to the 0.5% and 5% buffer, but similar or worse. Therefore the experiments were continued with the 0.5% and 5% buffers.

In test 3 and 4 the effect of the measurement time was tested. The 0.5% buffer yielded a higher BACTcontrol enzymatic activity (per enterococci) compared to the 5% buffer. Often, a measurement time of 60 minutes yielded higher enzymatic activity compared to the 120 minutes measurement time. However, sometimes the opposite was observed (120 minutes yielding higher activity compared to 60 minutes).

To test the effect of the presence of higher enterococci numbers and the amount of particles that may interfere with the measurements, a dilution series was performed in test 5. Increasing the number of enterococci and particles almost 10 and 100 fold (6 – 600 – 5000 cfu/vol) did not lead to a parallel increase in enzymatic activity (20.9 – 125.2 – 858.1 pmol). This indicated that at either higher numbers of enterococci or at a higher level of contamination the BACTcontrol enzymatic reaction is inhibited.

In addition, the enzymatic activity was higher at 60 minutes measurement time than at 120 minutes. This was also shown in test 7.

Several dilution series have been performed using the normal buffer with 0.5% or 5% Triton X-100 and 120 minutes incubation time: test 5 in Table 9 and two additional dilution series in Table 10.

These results are visualized in Figure 9 and show a good dose-response curve that is comparable for all three tests. Tests 1 and 2 were performed on the same WWTP effluent on two consecutive days, test 5 was performed weeks later on fresh WWTP effluent.

TABLE 10. MEASUREMENT TIME: 120 MINUTES, NORMAL BUFFER, SUBSTRATE: SIGMA.

	BACTcontrol (volume tested ml	Enzymatic activity BACTcontrol pmol	Enterococci culture cfu/vol	Enterococci culture MPN/vol	Activity/ enterococ pmol/cfu
Test 1					
Normal	10 ml (1:10 diluted)	3.9	24	23	0.16
Normal	100 ml (1:10 diluted)	26.8	160	335	0.17
Normal	1000 ml (1:10 diluted)	370.4	2000	1887	0.19
Test 2					
Normal	10 ml (1:10 diluted)	4.4	22	31	0.20
Normal	100 ml (1:10 diluted)	28.4	195	207	0.15
Normal	1000 ml (1:10 diluted)	308.2	1550	2008	0.20

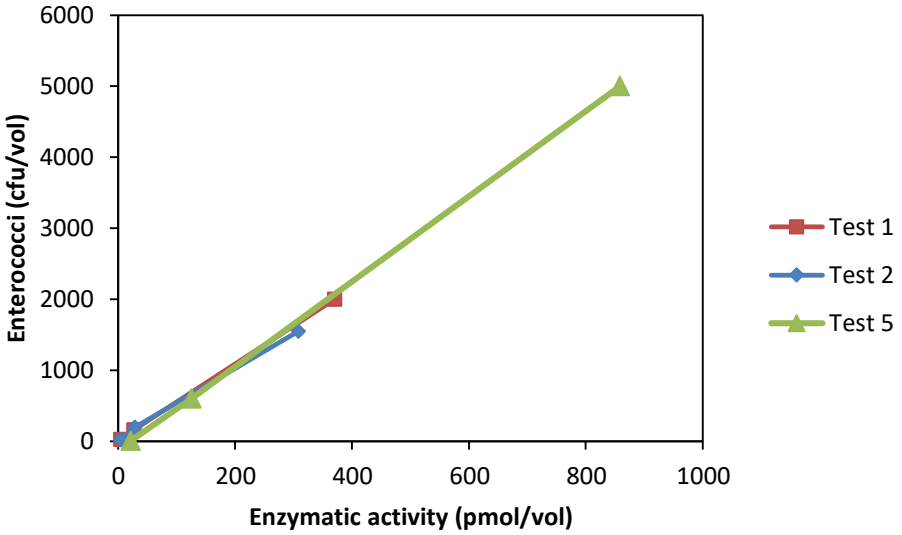

FIGURE 9. DILUTION SERIES OF WWTP EFFLUENT MEASURED WITH THE BACTCONTROL. NORMAL BUFFER WITH 0.5% OR 5% TRITON X-100, MEASUREMENT TIME: 60 OR 120 MINUTES. DETAILS OF MEASUREMENTS ARE DEPICTED IN TABLE 10 (TEST 1 AND 2) OR IN TABLE 9 (TEST 5).

In conclusion:

- Several lysis buffers are able to open or lyse enterococci in such a way that they become unculturable, however, only a few buffers are useful in combination with the BACTcontrol. Most of the tested buffers inhibit the enzymatic conversion of the substrate in some way, leading to lower enzymatic activity results in the BACTcontrol compared to the normal buffer. This indicates that the buffer treatment should balance lysis/permeabilising enterococci effectively, while keeping the enzymes intact and without inhibiting the substrate conversion.
- The normal buffer with 0.5% or 5% Triton X-100 and a measurement time of 60 minutes yielded the highest BACTcontrol signal. However, this buffer does not lyse all enterococci and may miss enterococci that are insufficiently open to substrate.
- Rinsing the filtration chamber with ultrapure water, after filtration of the water sample, increases the enzymatic activity showing that inhibiting compounds can be present in (surface) water samples.
- Dilution series of one water sample shows a good correlation between enzymatic activity and the number of culturable enterococci that are present within that one water sample.

Based on these tests it was decided to continue all experiments with the normal buffer (chapter 2.3.2.3).

2.5 Conclusions

- The Sigma-substrate performed better than the Aeser-substrate and was selected for subsequent experiments.
- Rinsing of the reaction chamber with ultrapure water before the enzyme reaction improved the BACTcontrol signal with a factor of approx.1.5.

- The difference in enzymatic activity of 25 mM or 100 mM substrate on measuring surface water is 7-56%. 25 mM was chosen as adequate and save costs, and used for all upcoming experiments.
- The normal buffer with 0.5% or 5% Triton X-100 and a measurement time of 60 minutes yielded the highest BACTcontrol signal. 0.5% was selected for future experiments.

3 Secondary optimization of the BACTcontrol enterococci assay (Het Waterlaboratorium)

3.1 General introduction and study aim

In the first optimization, experiments were conducted at KWR with water from two surface water locations. These resulted in optimized conditions for the BACTcontrol enterococci assay. This set of optimized conditions was taken and further evaluated in a secondary optimization at Het Waterlaboratorium. The experiments performed at Het Waterlaboratorium were targeted on answering the following questions:

- The substrate concentration was reduced from 100 to 25 mM (chapter 2). What is the influence of an even lower concentration of substrate (12.5 mM) on the measurement of enterococci with the BACTcontrol?
- Rinsing the BACTcontrol reaction chamber was shown to enhance the BACTcontrol signal by approx. 1.5x. In this secondary optimization, the effect of rinsing was tested in more and other water samples, Can differences in results between different (surface) water types, in relation to the number of culturable enterococci, be prevented by using an extra rinsing step in the protocol?
- For application in drinking water, the detection limit of the BACTcontrol enterococci assay was too high. Can the detection limit be lowered by filtering and analyzing a larger sample volume with the BACTcontrol?
- For application in drinking water, it is important to establish that the normal/ background (absence of enterococci) signal of the BACTcontrol is consistently low. What is the normal background signal of the BACTcontrol when tap water is measured?

3.2 Materials and methods of experiments

The BACTcontrol was tested at Het Waterlaboratorium for about 2 months. In this period, several protocols were performed using surface water samples from different locations. The BACTcontrol was used in an off-line setting at the laboratory.

During the incubation phase of the BACTcontrol's measurement process the fluorescence sensor is set to register a certain (n) amount of raw data points (expressed in relative fluorescence units (RFU)) per time. For each of these raw data points, the software will calculate the slope of the increase of RFU per minute, the average slope and the standard deviation of each the calculated slopes (calculated as deviation = (slope n – slope average)^2. The average slope and average standard deviation is calculated from these n values, usually discarding the first data points, since they markedly deviate from the average slope. The square root of this average standard deviation is multiplied by 3 and divided by the pmol conversion factor and this is the detection limit (in pmol/min/sample volume). According to the normal distribution the 3 sigma limit should contain 99,6% of the measurements/ total population.

Surface water of different locations was used (Figure 10): river water (Afgedamde Maas and Amsterdam Rijnkanaal) and a lake (Lake IJsselmeer). Surface water samples were collected in 10 liter jerry cans and transported under cold conditions (5 ± 3 ˚C) to Haarlem. The jerry can was placed in a vessel containing a layer of ice. A sample tube from the BACTcontrol was placed into the jerry can. Measurements on the surface waters started around 15:00. Before these measurements always a blank measurement was performed on tap water (source water: surface water) directly from a drinking water company. To test the effect of rinsing, the BACTcontrol measurements either included a rinsing step with 20 ml of MQ water or not. To test the effect of substrate concentration, the BACTcontrol signal with12.5 mM substrate concentration was compared with 25.0 mM on the same samples. The BACTcontrol was programmed to automatically analyze two sample volumes (Table 11). After the surface water measurements on Thursday, the BACTcontrol was connected to tap water using an overflow tank. From each of the surface water samples, the number of culturable enterococci was determined according to the NEN ISO 7899-2.

FIGURE 10. LOCATION OF SAMPLING LOCATIONS OF SURFACE WATER

TABLE 11. ANALYSIS SCHEDULE OF ENTEROCOCCI MEASUREMENT WITH THE BACTCONTROL ON SURFACE WATER.

Sample location	Sampling day	Period of measurement	Sample volume (ml)
Amsterdam Rijnkanaal	Monday	Monday 15:00 - Tuesday 12:00	100 and 1000
Afgedamde Maas	Tuesday	Tuesday 15:00 – Wednesday 12:00	100 and 1000
Lake IJsselmeer	Wednesday	Wednesday 15:00 - Thursday 12:00	50 and 100

3.3 Results

3.3.1 Substrate concentration

On the same water sample, BACTcontrol analyses were performed using a substrate concentration of 12.5 mM or 25 mM. Figure 11 presents the results of the BACTcontrol. The red line indicates when there are no differences between measurements on the same water sample with two substrate concentrations.

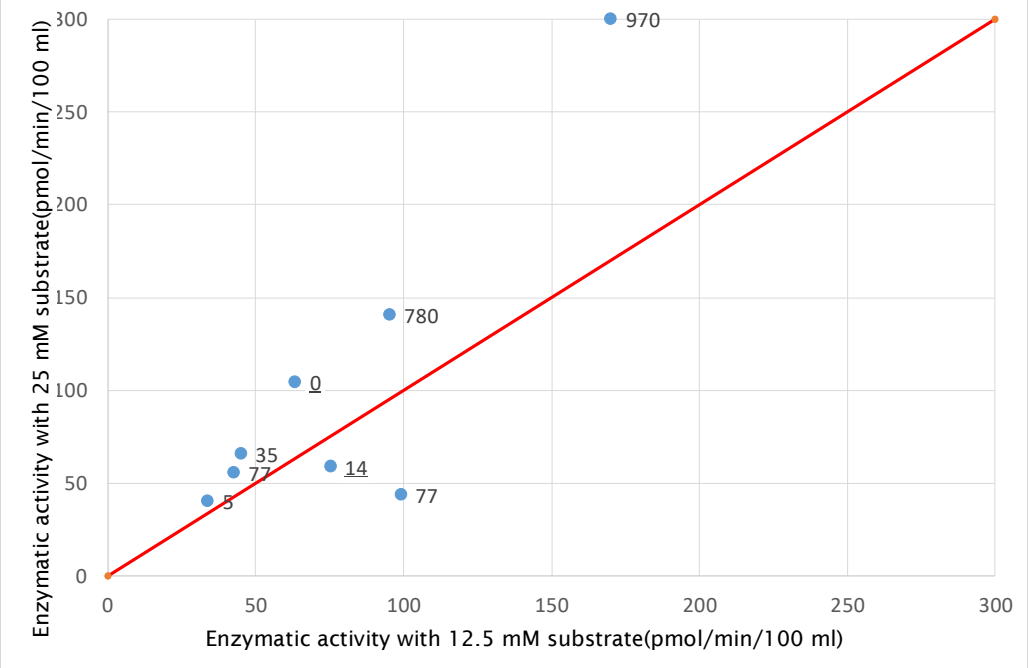

FIGURE 11. COMPARISON OF BACTCONTROL RESULTS USING TWO DIFFERENT SUBSTRATE CONCENTRATIONS. THE NUMBERS OF ENTEROCOCCI (CFU/100 ML) PRESENT IN THE WATER SAMPLE (CULTURE ANALYSES) IS PLOTTED WITH EACH DATA POINT.

When the enzymatic activity measured with the BACTcontrol was below 50 pmol/min, the results of the two substrate concentrations were comparable. Between 50 and 100 pmol/min/100 ml more variability was observed. When the enzymatic activity was higher than 150 pmol/min/100 ml, a 12.5 mM substrate concentration yielded a lower enzymatic activity than when the same water sample was measured with a 25.0 mM substrate concentration. This indicated that in the 12.5 mM assay, the substrate could be depleted at high enterococci numbers.

3.3.2 Extra rinsing step

In the previous project it was found that the enzymatic activity of the water from Lake IJsselmeer was higher compared to river water samples, even when the enterococci count with the conventional culture method was very low. One option is that free enzymes or chemical substances that can degrade the substrate were present in the lake water and thus produced a false positive signal. Here the effect of an extra rinsing step, to remove any compounds or free enzymes, was tested. The reaction chamber and filter were rinsed directly after filtering the water sample. Multiple water samples were taken from two sites (Lake IJsselmeer and Afgedamde Maas) on different days.

On the same water sample BACTcontrol analyses were performed with or without the rinsing step (20 ml MQ water; Figure 12).

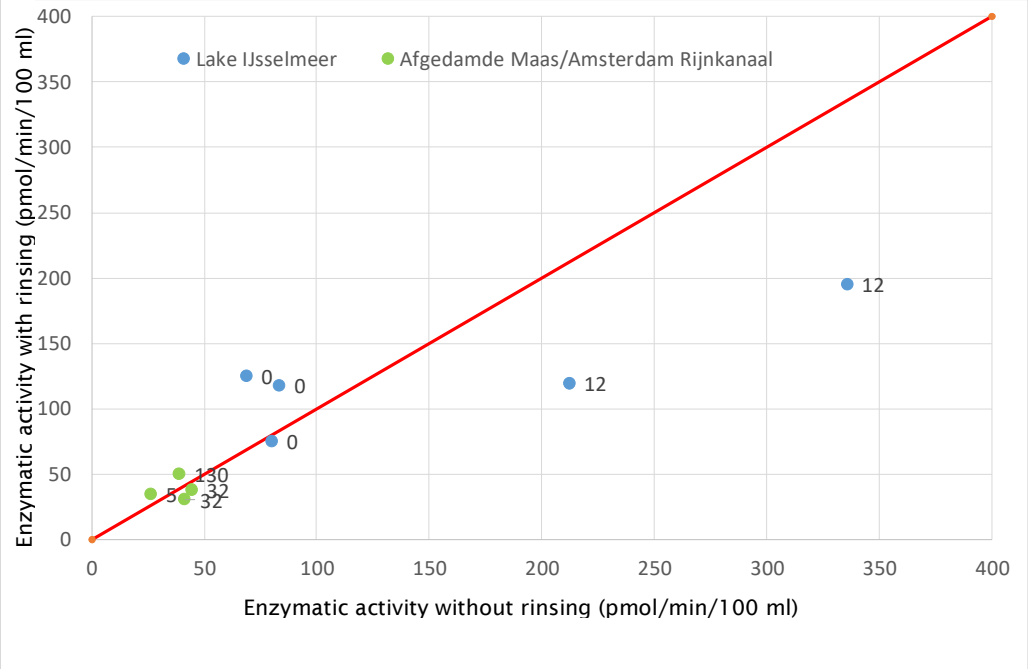

FIGURE 12. COMPARISON OF RESULTS OF BACTCONTROL WITH OR WITHOUT THE USE OF A RINSING STEP. THE NUMBERS OF CULTURABLE ENTEROCOCCI (CFU/100 ML) PRESENT IN THE WATER SAMPLE (CULTURE ANALYSES) IS PLOTTED WITH EACH DATA POINT.

The red line indicates when there are no differences between measurements with and without rinsing.

For the river water samples from Afgedamde Maas, there was no change in signal when the measuring chamber and the filter was rinsed with MQ water. This is also the case for one sample from Lake IJsselmeer, two samples showed higher signal with rinsing and two samples showed lower signal with rinsing. Most water samples from Lake IJsselmeer contained no culturable enterococci; nevertheless the enzymatic activity measured with the BACTcontrol was high (about 70 – 130 pmol/min/vol), regardless of whether a rinsing step was applied. These enzymatic activities are high in comparison to samples from Afgedamde Maas (< 50 pmol/min/vol).

The ß-glucosidase enzyme, of which the activity is measured in the BACTcontrol, is also used in the test kit Enterolert of IDEXX. Three samples of Lake IJsselmeer were tested with the BACTcontrol, culture and Enterolert (Table 12). The Enterolert yielded similar results as the cultivation method: low (0 – 4 per 100 ml) concentrations of culturable enterococci, while the enzymatic activity was very high. The cause of the higher activity in the BACTcontrol remained unclear.

TABLE 12. CULTURE, ENTEROLERT AND BACTCONTROL RESULTS OF WATER SAMPLES FROM LAKE IJSSELMEER.

	Culture	Enterolert		BACTcontrol
Sample date	CFU/100 ml	MPN/100 ml	Confidence level	pmol/min
1-4-2019	0	4.1	1.7-9.5	210
7-4-2019	1	1	0.0-3.7	140
15-4-2019	1	1	0.0-3.7	75

3.3.3 Filtration volume to lower the detection limit

Figure 13 presents the result of the BACTcontrol for water samples obtained at the Afgedamde Maas compared with the enterococci number measured with the culture method. Also the detection limit of the instrument is presented.

The number of enterococci measured with culture was not correlated to the enzymatic activity measured with the BACTcontrol, at culturable enterococci numbers between 6 and 130 the enzymatic activity of the BACTcontrol is fairly stable around 40 pmol/min/100 ml and does not increase with higher culturable enterococci numbers. A clearly increased BACTcontrol signal was observed in the sample in which the number of culturable enterococci is 1000 cfu/100 ml.

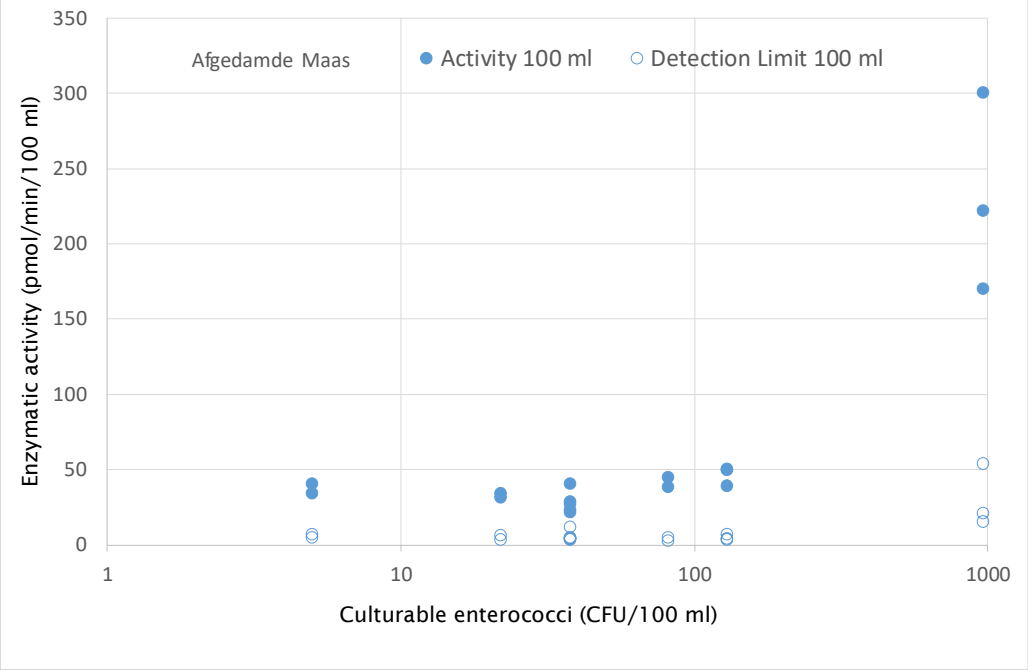

FIGURE 13. RESULTS OF THE BACTCONTROL ON 100 ML WATER SAMPLES OF AFGEDAMDE MAAS IN RELATION TO THE NUMBER OF CULTURABLE ENTEROCOCCI.

In Figure 14 results are presented when 1000 ml of sample is provided to the filter of the BACTcontrol compared to the standard 100 ml volume. In this figure it was assumed that the number of culturable enterococci (normally measured in 100 ml) can be multiplied with a factor of 10 to determine the number of culturable enterococci in 1000 ml.

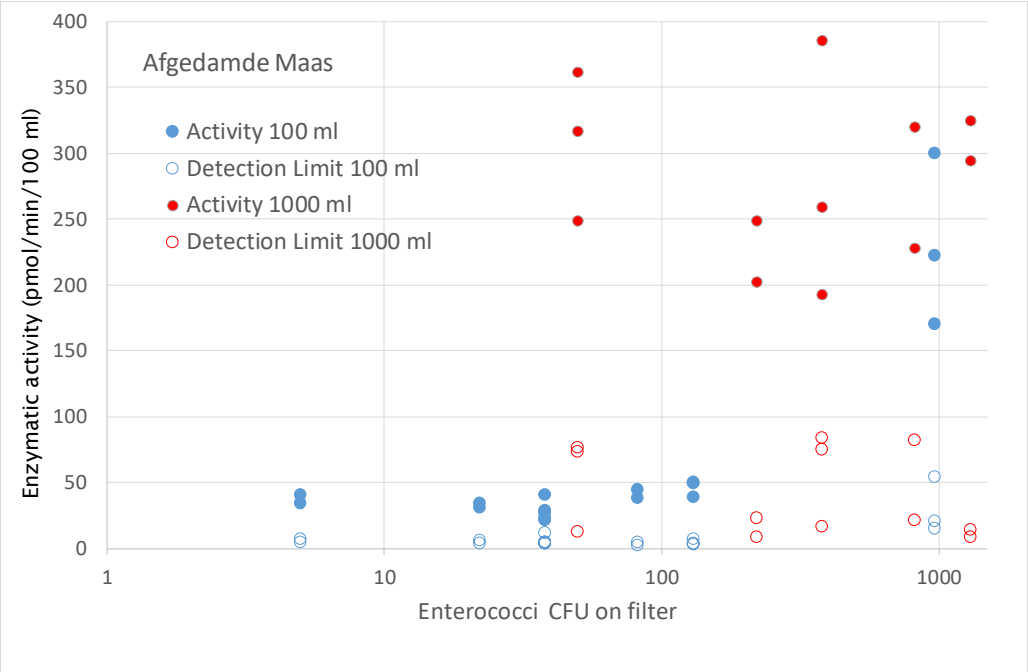

FIGURE 14. BACTCONTROL MEASUREMENTS OF 100 ML AND 1000 ML SAMPLES FROM AFGEDAMDE MAAS IN RELATION OF NUMBER OF CULTIVATED ENTEROCOCCI.

Figure 14 shows that an increase in the filtered sample volume led to an increase of the detection limit presented by the BACTcontrol and the signal-to-detection limit ratio improved. An increase in the number of culturable enterococci did not lead to a consistent increase in the enzymatic activity measured by the BACTcontrol. So for Afgedamde Maas samples, it was not apparent that when more sample volume (1000 ml instead of 100 ml) is provided to the BACTcontrol, the limit of detection improved. Although the signal-to-detection limit did increase, no increase was observed with increasing culturable enterococci numbers, so we cannot discriminate between background surface water signal and enterococci signal in these samples.

3.3.4 Background signal BACTcontrol with tapwater

In addition to measurement of surface water with the BACTcontrol, also tap water was measured during the weekends in an online setting (Figure 15).

The enzymatic activity of tap water was below the enzymatic activity from surface water and in most cases below the detection limit of the BACTcontrol. The increase in enzymatic activity that was measured at the end of January/beginning of February 2019, was possibly caused by formation of biofilm in the sample tubing or due to carry-over from previous measurements when surface water was used. After this observation, all tubes were rinsed with 70% ethanol and tap water after measuring surface water and before a BACTcontrol measurement was performed on tap water.

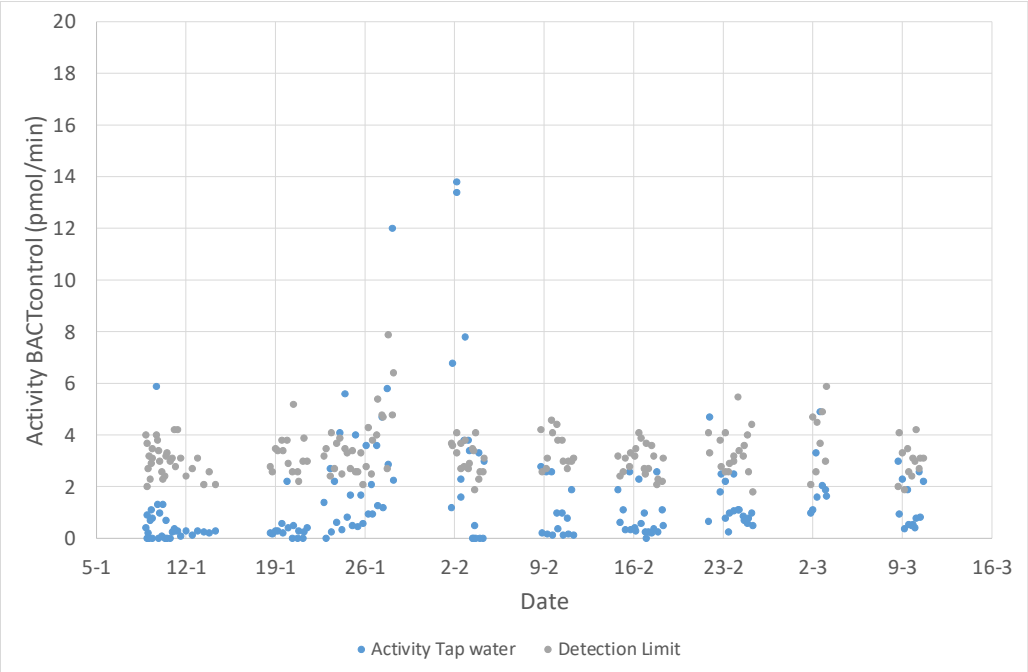

FIGURE 15. MEASUREMENT OF TAP WATER (100 ML)WITH THE BACTCONTROL ENTEROCOCCI ASSAY AT LOCATION HAARLEM DURING THE WEEKENDS.

3.4 Conclusions

There was no difference in enzymatic activity when 12.5 mM or 25 mM substrate was used in the BACTcontrol for measurements of surface waters below 50 pmol/min. Between 50 and 100 pmol/min more variability was observed. When the enzymatic activity exceeds 150 pmol/min/vol (about 1000 culturable enterococci), the use of 25 mM substrate is preferred to prevent depletion of substrate during the incubation in the reaction chamber of the BACTcontrol.

Use of an extra rinsing step, after filtration of the water sample by the BACTcontrol, did not improve the enzymatic activity of the BACTcontrol. In samples of Lake IJsselmeer, high enzymatic activities were measured (higher than in samples of Afgedamde Maas) while the water contained no or very low numbers of culturable enterococci. An extra rinsing step did not improve this. This makes comparison of the enzymatic activity of this water type to other locations difficult and suggests that the BACTcontrol is not suitable for measuring this type of water. The cause of the higher activity in the BACTcontrol using water from Lake IJsselmeer remains unclear.

A larger sample volume (1000 ml instead of 100 ml) improved the signal-to-detection limit ratio of the BACTcontrol enterococci assay, but the results of Afgedamde Maas did not show an improvement in the BACTcontrol signal-to-culturable enterococci relation: the BACTcontrol signal did not increase with increasing culturable enterococci concentrations.

The background signal of the BACTcontrol when monitoring drinking water in an on-line setting is low (0 – 4 pmol/min).

4 Application of the BACTcontrol enterococci assay in a drinking water setting (Vitens Laboratory)

4.1 Introduction and aim

The Vitens Laboratory carried out the following tests to answer two research questions that resulted from the studies at Het Waterlaboratorium (chapter 3)):

- Expand the dose-response curve (BACTcontrol vs culture) by placing the BACTcontrol measuring surface water samples every week. Samples were taken at production site Epe and brought to the laboratory for measurement with the BACTcontrol. On the same samples the number of culturable enterococci was determined with the enterococci culture method (NEN ISO 7899-2).
- Compare the BACTcontrol in drinking water samples with the culture method and compare the enzymatic activity with the measurements at Het Waterlaboratorium and KWR. Samples were measured with the BACTcontrol and the enterococci culture method.

In addition, Vitens Laboratory collated user experience with the BACTcontrol in a water laboratory environment.

4.2 Materials and methods

The BACTcontrol was placed at the Vitens Laboratory for 5 weeks. Drinking water and surface water samples were measured with the BACTcontrol and compared with the results of the enterococci culture method. Every Tuesday, surface water from production site Epe was sampled and brought to the laboratory. On Wednesday the surface water samples were measured with the BACTcontrol and the culture method. On the other days drinking water samples were measured with the BACTcontrol and a few times with the culture method as well.

4.2.1 Water type and sampling location

For the experiments water samples from two locations were used. The first location is production site Epe (Figure 16). At this location surface water from ‘de Klaarbeek’ and ‘de Grift’, two small rivers, is infiltrated into the ground (soil passage). After extraction the water is used for drinking water production. Historical data show that the surface water that is used for infiltration contains high numbers of enterococci and is therefore suitable for tests with the BACTcontrol.

FIGURE 16. LOCATION OF PRODUCTION SITE EPE OF VITENS

The second location is the garage next to the Laboratory of Vitens, located in Leeuwarden (Figure 17). The tap water was used to perform the tests with drinking water. Here also the enterococci cultures were performed (NEN ISO 7899-2).

FIGURE 17. LOCATION OF VITENS LABORATORY IN LEEUWARDEN

The following water samples were used:

- Surface water from production site Epe
  - 100 ml
  - 500 ml
  - 1000 ml
- Drinking water from the Vitens Laboratory in Leeuwarden
  - 100 ml
  - 1000 ml

4.2.2 Experimental set-up

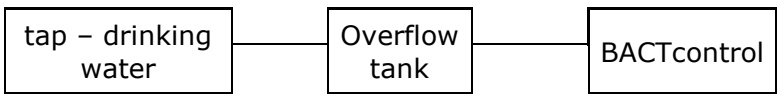

FIGURE 18. SCHEMATIC DRAWING OF THE CONNECTION OF THE BACTCONTROL

4.2.3 Details of sampling and measurements

In the normal set up (Figure 18) the BACTcontrol measured drinking water from the tap via an overflow tank. Once a week surface water from Epe was manually tested with the

BACTcontrol. The normal buffer and 25 mM substrate was used in each of the BACTcontrol measurements, No additional rinsing or cleaning procedures were performed, other than the standard sampling.

On most measurement days a blank measurement was performed on the BACTcontrol on drinking water.

4.2.3.1 Drinking water samples

A few times drinking water sample were taken (from the tap) for measurement with the culture method. The samples were taken at the same time as the 'filtration start time' of the BACTcontrol.

4.2.3.2 Surface water samples

Every Tuesday three samples were taken by the sample taker and transported to the laboratory (2 – 5 ˚C) in Leeuwarden. One sample-bottle was used for each of the three different filtration volumes (100 ml, 500 ml, 1000 ml). The samples were kept at 2 – 5 ˚C until the start of the measurement with the BACTcontrol and the culture method. The aim was to perform three measurements a day with all three volumes. Because of external factors, some measurements were performed one day later (Table 13).

4.3 Results

4.3.1 Comparison of BACTcontrol with culture in surface water samples

Table 13 and Figure 19 show the results and the correlation between the enzymatic activity measured with the BACTcontrol and the enterococci counts with the culture method on the surface water samples. There seems to be only a limited correlation between the two methods; although the p-value is below 0.05 the fit of the trend line through all data points is mediocre (R^2 =0.51). The variability of the signal of the BACTcontrol at the same concentration of culturable enterococci can be very substantial (Figure 19); the p/mol-cfu ratio varied between 1 and 10.8 (Table 13).

Table 13 shows all the results measured with the BACTcontrol and the culture method of the surface water samples. Some of the samples were measured two or three times with the same filtration volume with the BACTcontrol and once with the culture method. This is shown with *. The results from these replicate samples did not differ much from each other (between the lowest and highest value max. 15%), showing that the results from the BACTcontrol on a single water sample were reproducible.

4.3.1.1 Water matrix

There are substantial differences in the relation between BACTcontrol enzymatic activity and culturable enterococci in the surface water of Epe between different sampling days (Table 13), but multiple analysis of the same volume on the same day produced comparable BACTcontrol outputs. The pmol/cfu values are higher than measured at KWR and HWL. It is unclear what caused the differences, possibly the water matrix had an effect.

The drinking water samples give low values with the BACTcontrol and a zero value for the samples measured with the culture method (Table 15).

TABLE 13. COMPARISON OF BACTCONTROL AND ENTEROCOCCI CULTURE OF SURFACE WATER FROM PB. EPE. GIVEN IS THE DATE AND TIME OF THE START OF THE MEASUREMENTS, WITH SAMPLE VOLUME TESTED IN BACTCONTROL AND ON CULTURE, THE RESULT OF THE BACTCONTROL IN PMOL/MIN FOR THE TESTED SAMPLE VOLUME AND THE NUMBER OF ENTEROCOCCI COLONIES ON CULTURE PER 100 ML AND PER THE SAMPLE VOLUME. 1*, 2*, 3*: SAME SAMPLE. THE CALCULATED ENZYMATIC ACTIVITY (IN PMOL) PER CULTURED ENTEROCOCCI BACTERIA IS ALSO GIVEN.

Date	Time	Sample volume	Detection limit (pmol/min)	Enzymatic activity (pmol/min)	Enterococci culture cfu/100 ml	Enterococci culture cfu/volume	pmol/cfu
30-5-2019	18:09:54	1000 ml	584,2	1936,0	55	550	3,52
30-5-2019	13:44:42	500 ml	148,3	1291,6	37	185	6,98
30-5-2019	10:09:09	100 ml	24,3	186,0	38	186	1,00
23-5-2019	20:06:37	1000 ml	433,9	3131,2	65	650	4,82
23-5-2019	13:49:22	100 ml[1*]	19,9	240,1	73	73	3,29
23-5-2019	12:08:52	100 ml[1*]	20,2	280,6	73	73	3,84
23-5-2019	10:27:50	100 ml[1*]	36,7	265,8	73	73	3,64
22-5-2019	16:46:48	500 ml[2*]	37,5	914,4	128	640	1,43
22-5-2019	13:53:01	500 ml[2*]	32,9	1040,4	128	640	1,63
15-5-2019	19:32:42	1000 ml	57,9	1292,8	12	120	10,77
15-5-2019	13:50:50	500 ml	47,7	432,5	13	65	6,65
15-5-2019	10:35:31	100 ml	32,0	79,9	12	12	6,66
9-5-2019	1:07:43	500 ml[3*]	8,3	734,2	23	115	6,38
8-5-2019	22:29:56	500 ml[3*]	45,4	724,0	23	115	6,30
8-5-2019	19:59:57	500 ml[3*]	25,9	811,9	23	115	7,06
8-5-2019	17:05:04	100 ml	27,4	234,2	29	29	8,08
8-5-2019	12:07:18	1000 ml	83,7	947,1	30	300	3,16

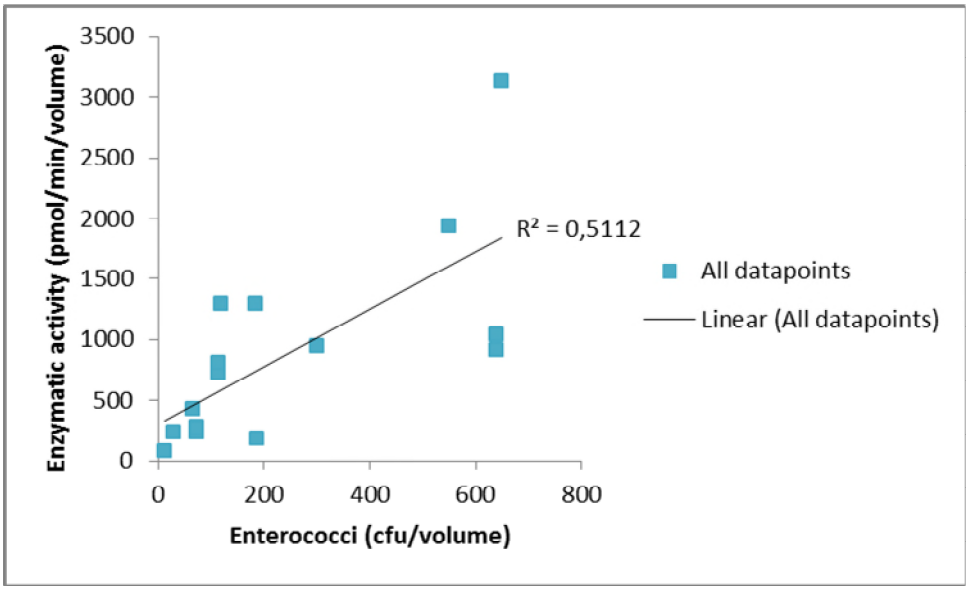

FIGURE 19. ENZYMATIC ACTIVITY (NET ACTIVITY) IN PMOL/MIN/VOLUME VS. ENTEROCOCCI CULTURE RESULTS IN CFU/VOLUME. SURFACE WATER OF PB. EPE WAS MEASURED WITH THE BACTCONTROL AND CULTURE AT THE SAME TIME. THE SAME VOLUME WAS USED IN BOTH METHODS.

4.3.1.2 Filtration volume

The results of the surface water do not correlate all the time. It seems that the biggest factor is the filtration volume.

The results with the BACTcontrol showed the highest correlation between the culture method at a filtration volume of 1000ml. The lower the filtration volume, the lower the correlation (Table 14).This is an indication that higher culturable enterococci levels (120 – 650) produce a better signal-to-noise ratio than lower levels.

TABLE 14. CORRELATION BETWEEN BACTCONTROL VS. CULTURE (SURFACE WATER) IN DIFFERENT SAMPLE VOLUMES

	1000 ml (n = 4)	500 ml (n = 4)	100 ml (n = 4)
Correlation surface water BACTcontrol/culture (R^2)	0.84	0.31	0.25

The enzymatic activity of drinking water, in the absence of enterococci, is consistently low (0.21 – 2.41 pmol/min/volume; Table 15). As all culture results turned out to be negative for enterococci, it cannot be said if and how much the enzymatic activity would increase upon the presence of a few enterococci bacteria.

TABLE 15. COMPARISON OF BACTCONTROL AND ENTEROCOCCI CULTURE OF DRINKING WATER FROM THE LABORATORY AT LEEUWARDEN. GIVEN IS THE DATE AND TIME OF THE START OF THE MEASUREMENTS, THE RESULT OF THE BACTCONTROL IN PMOL/MIN/VOLUME FOR THE TESTED SAMPLE VOLUME AND THE NUMBER OF CULTURABLE ENTEROCOCCI PER 100 ML

Date	Time	Enzymatic activity (pmol/min)	Sample volume (ml)	Enterococci culture cfu/100 ml
29-5-2019	14:11:08	0,26	1000	0
13-5-2019	16:15:42	0,49	1000	0
13-5-2019	14:03:37	0,55	1000	0
13-5-2019	11:51:25	0,57	1000	0
10-5-2019	13:56:25	0,21	1000	0
10-5-2019	11:44:17	0,26	1000	0
10-5-2019	9:32:05	0,43	1000	0
7-5-2019	13:22:53	1,12	1000	0
6-5-2019	16:39:54	0,96	1000	0
6-5-2019	13:44:48	1,07	1000	0
6-5-2019	10:08:51	2,41	1000	0

4.3.1.3 Cleaning step

The enzymatic activity of the first drinking water samples that had been analyzed after the surface water samples were high (Figure 20). No cleaning step was performed during these analyses. When measuring surface water, the enzymatic activity was high. The first drinking water samples after surface water samples give a high enzymatic activity. When measuring drinking water continued, the enzymatic activity was much lower. Therefore, after the second switch between surface water and drinking water, a

cleaning step was introduced that prevented the cross-contamination between surface water sample and drinking water sample (Figure 20). So, after a sample with high enzymatic activity is examined in the BACTcontrol a cleaning step is necessary before a sample with low enzymatic activity is tested, to prevent cross-contamination.

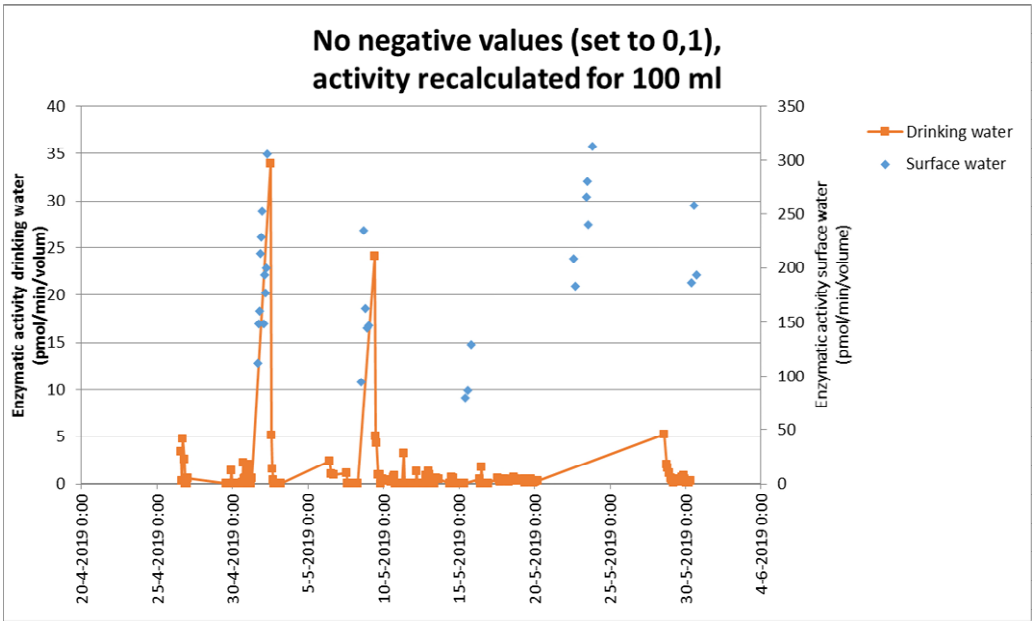

FIGURE 20. CONTAMINATION ON SAMPLES WITH LOW ENZYMATIC ACTIVITY FROM SAMPLES WITH HIGH ENZYMATIC ACTIVITY.

4.4 User experience with the BACTcontrol system

4.4.1 Positive:

- Robust
- Easy to use
- Adjust (parameter) settings; connect samples, refill chemicals and starting the BACTcontrol
- Volume registration system
- Signal problems/exceedance clear and alarms are easy to fix
- When you press stop, the system stops immediately. To quit the system, the current sample does not have to be finished.

4.4.2 Recommendations

- Larger volumes of chemicals, so refill is needed less quickly. The consumables were empty faster than expected.
- Being able to set different filtration volumes and time intervals.

4.4.3 Points for attention

- Little pressure on the inlet hose; this can cause problems in the field.
- Filtration is slow, especially with larger volumes.
- Cleaning the system takes a lot of time
- After measuring enterococci, the next blank will not give 0 as a value.
- It is not easy to relate the results to the culture results (cfu/100 ml). This makes it harder to communicate with process operators and other staff.

4.5 Conclusion

4.5.1 Conclusions

There correlation between the BACTcontrol signal and culturable enterococci was low in Epe surface water; although the p-value was below 0.05 the fit of the trend line through all data points is mediocre (R^2 =0.51). The variability of the signal of the BACTcontrol at the same concentration of culturable enterococci can be very substantial: the p/mol-cfu ratio varied between 1 and 10.8. The best correlation was found with a filtration volume of 1000ml. The lower the filtrated volume, the lower the correlation.

The BACTcontrol signal from the drinking water samples was low and the results with the culture method were zero. No inexplicable deviations have been observed. After a sample with a high enzymatic activity and before a sample with a low enzymatic activity, a cleaning step is necessary to prevent contamination.

The detection limit of the BACTcontrol is not as low as hoped. For the production of drinking water, Vitens uses groundwater in most cases. For both, the desired detection limit would be 1/100 ml for enterococci. Vitens has only a few production sites, which are under some influence from surface water, where higher detection limits could be appropriate.

4.5.2 Recommendations

- This part of the study showed the highest correlation between BACTcontrol signal and culturable enterococci for 1000 ml samples of surface water (Klaarbeek). The number of samples tested is low (n=4). It is therefore recommended to conduct further research to determine whether a sampling volume of 1000 ml improves the correlation between the BACTcontrol and the culture method from surface water from other sites.

- The detection limit of the BACTcontrol enterococci assay is too high for application for regulatory drinking water monitoring. It is recommended to determine how the detection limit can be reduced.

5 Application of the BACTcontrol enterococci assay at field sites (Hoogheemraadschap van Delfland and Het Waterlaboratorium)

5.1 Introduction

The BACTcontrol is designed for deployment locations for semi-continuous, on-site monitoring. In this part of the study, the BACTcontrol was placed in the field at two bathing beaches (Hoogheemraadschap van Delfland) and one site where surface water is abstracted for the production of drinking water (Het Waterlaboratorium) to obtain experience with the performance of the BACTcontrol for field testing and to compare the results of the BACTcontrol and culture enterococci assay in these field settings. For these sites, the BACTcontrol was equipped with the smaller (2ml) reaction chamber.

5.2 Water playground Buytendelft

5.2.1 Overview of Buytendelft

Buytendelft is a water playground situated in the North of Delft. In the water playground a continuous flow of surface water is used. It is an official bathing water; the Hoogheemraadschap van Delfland (Water Board) measures the water quality (Escherichia coli and intestinal enterococci with culture methods) every other week during the bathing season. For many years there are bacterial problems (high peaks of *E. coli* and Intestinal Enterococci) due to several causes. In Figure 21 an overview of the situation in Buytendelft is given.

FIGURE 21. OVERVIEW OF THE BUYTENDELFT WATER PLAYGROUND

The blue arrows in Figure 21 show the direction of the surface water flow through the playground. The total volume of the playground is approximately 400 m3. It takes around six hours to renew the water in the total system. Bacterial problems are caused by birds, a high density of children playing in the water and sometimes the surface water is polluted at in the inlet (in times of heavy rainfall).

5.2.2 Application of the BACTcontrol at Buytendelft

The BACTcontrol system was placed at the water playground for two weeks in September 2018. The system was placed next to the water and samples were taken directly in the water system. Figure 22 shows an overview of the situation.

FIGURE 22. THE BACTCONTROL AT THE BUYTENDELFT WATER PLAYGROUND

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The monitoring program of Delfland shows that values of Intestinal Enterococci in the range of 30 – 1400 n/100ml were found by the enterococci culture method (NEN ISO 7899-1). Because of the high levels of enterococci, the BACTcontrol was programmed to take samples of 100 ml every three hours. During the experiment four samples a day were taken, which were send to the laboratory Aquon for the enterococci culture method using microtiter plates using 1:2 and 1:20 dilutions and MPN tables (NEN ISO 7899-1).

Due to filtering problems (there are sand particles in the water) it took often more time than 3 hours for the analysis with the BACTcontrol. The filters have been changed a few times because the system was totally blocked.

5.2.3 Results

In Table 16 the results of the experiment with the BACTcontrol are given. In the first column the date and time of sampling are given, in the second column the results of the BACTcontrol and in the third column the results of the laboratory analyses are given.

TABLE 16. RESULTS OF THE BACTCONTROL EXPERIMENT VERSUS CULTURABLE ENTEROCOCCI.

Date/time	BACTcontrol pmol/min/100 ml	Enterococci culture n/ 100ml	Date/time	BACTcontrol pmol/min/100ml	Enterococci culture n/100ml
19-09-18 20:00	453,2		25-09-18 1:59	1195,9	
19-09-18 23:00	397,3		25-09-18 7:59	1951,2	220
20-09-18 2:00	288,0		25-09-18 10:59	2830,8	270
20-09-18 8:00	729,5	77	25-09-18 14:00	759,9	140
20-09-18 11:00	977,1	140	25-09-18 16:59	808,6	94
20-09-18 14:00		77	25-09-18 19:59	2424,8	
20-09-18 17:00		140	25-09-18 22:59	878,4	
20-09-18 20:00	845,3		26-09-18 1:59	1913,2	
20-09-18 23:00	757,9		26-09-18 8:00		61
21-09-18 8:00	956,6	350	26-09-18 10:59	3467,8	15
21-09-18 11:00	512,0	460	26-09-18 14:00	949,8	30
21-09-18 14:00	799,2	390	26-09-18 16:59	566,2	15
21-09-18 17:00	938,9		26-09-18 19:59	1300,2	
21-09-18 20:00	1088,7		27-09-18 8:00		45
21-09-18 23:00	965,1		27-09-18 11:00		45
22-09-18 2:00	1353,0		27-09-18 14:00		77
22-09-18 8:00	1780,7		27-09-18 16:59	1012,2	180
22-09-18 11:00	1694,0		27-09-18 19:59	1429,4	
22-09-18 14:00	1057,4		27-09-18 22:59	575,2	
22-09-18 17:00	1580,8		28-09-18 1:59	729,4	
22-09-18 20:00	1195,8		28-09-18 7:59	1533,4	200
24-09-18 8:00		270	28-09-18 10:59	925,4	220
24-09-18 11:00		250	28-09-18 14:00	827,3	77
24-09-18 14:00		230			
24-09-18 16:59	3969,3	300			
24-09-18 19:59	705,2				
24-09-18 22:59	1277,4				

Due to problems with the filter in the BACTcontrol there are no results during the daytime at 20, 24, 26 and 27 September. The filter was blocked many times and the sampling times was longer than expected. The samples for the lab analysis were taken during the daytime and not at night or in the weekend.

In Figure 23 the results for the BACTcontrol are given. There is a large variation in the results, 288 – 3969 pmol/min/100ml. The laboratory analyses give results in the range of 15-460 n/100ml. Because there is a continuous flow of surface water, changes in the

bacterial concentration can occur. During the experiment the weather was dry with temperatures of 13 – 17 °C and there were no children playing in the water.

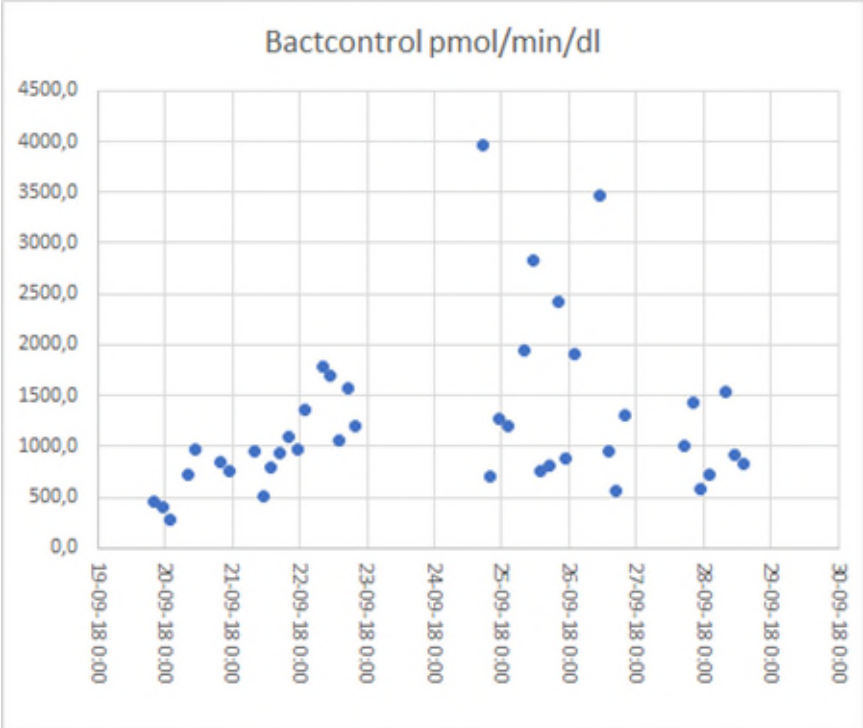

FIGURE 23. THE RESULTS OF THE THE BACTCONTROL AT THE BUYTENDELFT WATER PLAYGROUND.

In Figure 24 the results of the lab method are plotted versus the results of the BACTcontrol. The correlation between the two methods in this experiment is very poor.

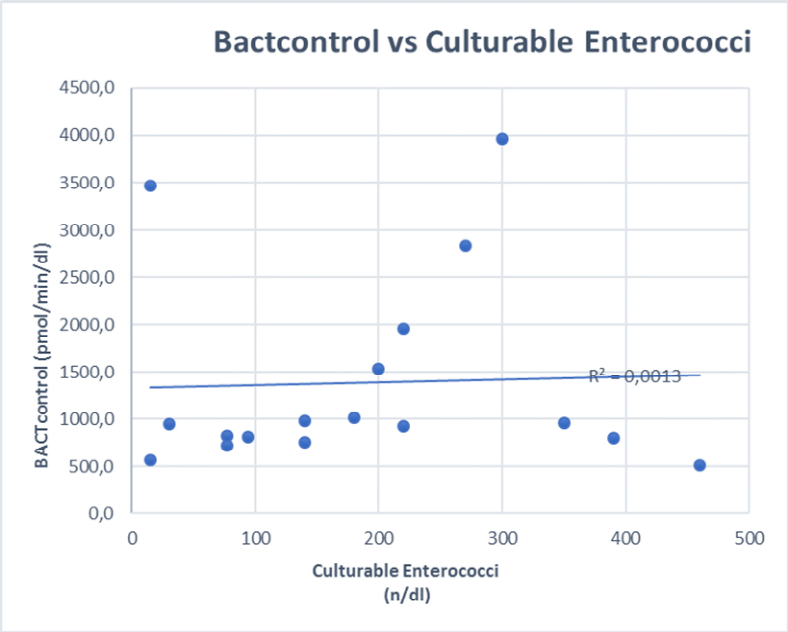

FIGURE 24. THE RESULTS OF THE THE BACTCONTROL VERSUS CULTURABLE ENTEROCOCCI.

5.2.4 Conclusions

- The correlation between the results of the BACTcontrol and lab analysis is very low in this experiment;
- Direct sampling at this water playground gives problems with the BACTcontrol because of the sand particles in the water;
- A lot of measurements could not be used for comparison because the sampling time differs.
- The bacterial concentration level of water playgrounds in surface water is much higher than the levels of drinking water.

5.2.5 Recommendations

- Repeat the test in another water playground where no sand particles are in the water.
- Take samples at the same time for better comparison of the culture method and BACTcontrol.
- Because of the high bacterial levels and BACTcontrol signal it is advised to decrease the sample volume of the BACTcontrol.

5.3 Water playground Tanthof

5.3.1 Overview of water playground Tanthof

Tanthof is a water playground situated in the south of Delft. In the water playground a continuous flow of surface water is used. The water is pumped in from the surrounding waters. It is an official bathing water where the Hoogheemraadschap van Delfland (Water Board) measures the water quality (Escherichia coli and intestinal enterococci with culture methods) every other week during the bathing season. For many years there are bacterial problems (*E. coli* and Intestinal Enterococci) due to several causes.

In Figure 25 an overview of the situation in Tanthof is given.

FIGURE 25. OVERVIEW OF THE TANTHOF WATER PLAYGROUND.

The red arrows in the figure show the direction of the surface water flow through the playground. The water is pumped from the east ditch and at the water playground it is divided into two streams. One part is a natural water stream with a sandy bottom. The other part is a plastic gutter that discharges in a plastic pond. The total volume of the playground is approximately 300 m^3. It takes around four hours to renew the water in the total system. Bacterial problems are caused by birds, a high density of children playing and often the surface water is polluted at the inlet. A lot of dogs are taken out around the playground and by heavy rainfall faeces of dogs run off into the surface water.

The playground contains less sand than Buytendelft. So less operational issues with the BACTcontrol were expected. There are three sampling points (yellow marks in Figure 25), number 017 in the pond where most of the children are playing, number 024 at the inlet of the playground and number 05 in a ditch with inlet water that is coming out of the nearby polder. Historical data show that the ditch from the polder (no. 05) has much lower Enterococci values than the other two points. At the official sample point (pond, no. 17) values of Intestinal Enterococci were found in the range of 77 – 2900 n/100ml during the swimming season of 2019.

5.3.2 Application of the BACTcontrol in Tanthof

The experiment of the BACTcontrol in Tanthof took place at August 28th until September 5th 2019. The weather conditions were good, sunny weather with temperatures of 25-30 °C. During the daytime, a lot of children were playing in the water, especially in the weekend.

The BACTcontrol was placed in a closed room and during the day time (8:00 – 16:00) the manager of the playground took a sample of 1 liter every two hours. The sample was divided in two parts. One bottle was filled and stored in a fridge for the enterococci culture method (NEN ISO 7899-1) that was conducted as described in 5.2 at Aquon laboratory. The other bottle was placed in the BACTcontrol for direct analyses. Samples were taken from three different points (05, 17 and 24) to test different concentration levels. At the beginning of the experiment samples volumes of 100 ml were used in the BACTcontrol. The filtration time in the BACTcontrol became more than 60 minutes and samples showed a very high activity 754 – 1931 pmol/min/100ml. The next day the sample volume was reduced to 20ml and the filtration time reduced to 5-10 minutes. This volume was used throughout the rest of the experiment.

In Figure 26 some pictures of the water playground are given.

5.3.3 Results

The experiment was set up to take samples from the pond (017) at 8:00, 12:00 and 16:00 to follow the bacterial activity during the day time. At 10:00 a sample was taken from the inlet water and at 14:00 a sample from the polder ditch. The results of the experiment are given in Table 17. In the last column, the sampling point is given.

FIGURE 26. PICTURES OF THE TANTHOF WATER PLAYGROUND.

TABLE 17. RESULTS OF THE BACTCONTROL EXPERIMENT VERSUS CULTURABLE ENTEROCOCCI AT TANTHOF.

Date	Time	BACTcontrol enzyme activity pmol/min/20ml	Enterococci culture n/100ml	Sampling point
30-aug	8:00	270	77	017
	10:00	296	200	024
	12:00	512	94	017
	14:00	168	61	05
	16:00	236	94	017
31-aug	8:00	280	110	017
	14:00	436	580	05
	16:00	327	Not reliable	017
1-sep	8:00	250	220	017
	10:00	415	250	024
	12:00	517	160	017
2-sep	8:00	301	530	024
	10:00	344	370	017
	12:00	282	160	017
	14:00	162	94	05
	16:00	507	370	017
3-sep	8:00	229	77	017
	10:00	371	140	024
	12:00	343	250	017
	14:00	258	46	05
	16:00	325	250	017
4-sep	8:00	184	290	017
	10:00	296	270	017
	10:00		160	017
	12:00	328		017
	14:00	382	420	017
	16:00	292	250	017
5-sep	8:00	254	520	017
	10:00	292	460	017
	12:00	431	270	017
	14:00	419	330	017
	16:00	313	250	017

In Figure 27 the results of the BACTcontrol and the laboratory analyses are plotted for the sample point pond (no. 17).

Data from before 30/8 were excluded since the culture method was applied on samples older than 24h. On the first three days a lot of children were playing in the water because it was the last weekend of summer holidays. In Figure 27 it is clear that in the morning the activity started at values around 250 pmol/min/20 ml and increased in the afternoon to values of 500 pmol/min/20ml. The observations of the local bathing water manager that visitor counts increased as the day progressed fits to the BACTcontrol measurements. The culture method does not show that pattern (see for instance Sep 5 where the 8 and 10am counts are high, when observed visitor numbers (data not

recorded) were low). On Monday September 2nd the playground was closed for cleaning. The next days not many children were playing because the schools had started. On these days the bacterial activity was increasing to levels of 400 pmol/min/20ml.

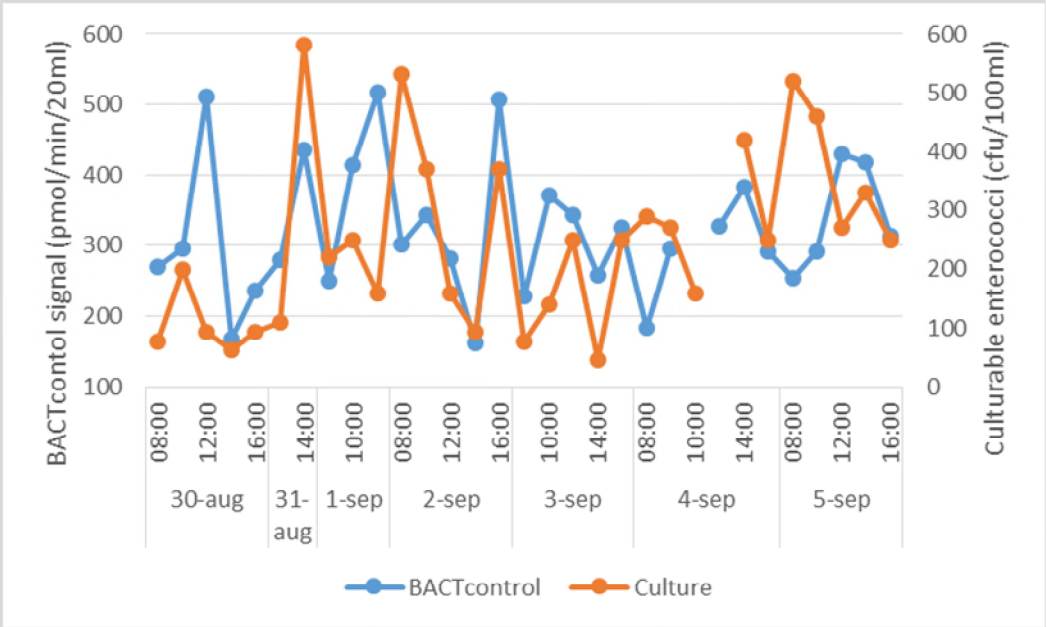

FIGURE 27. RESULTS OF THE BACTCONTROL AND CULTURABLE ENTEROCOCCI AT SAMPLE POINT 17 (POND).

The laboratory analyses followed the same trend as the BACTcontrol, but the increasing culturable enterococci concentration is not always paralleled by an increase in the BACTcontrol signal (see Figure 27). This was expected, because samples were taken at the same site at the same time.

On August 31st the BACTcontrol did measure three times the same sample, because the manager was too busy to change the sample. The results are listed in Table 18. The three measurements have a standard deviation of 6.5%, which is very good. On September 4th two sub-samples were taken from the sample and sent to the laboratory. The two results give a standard deviation of 40%. The laboratory Aquon gave information that the standard deviation for bacterial analysis, like Intestinal Enterococci, is about 35 %.

TABLE 18. RESULTS OF ANALYSING THE SAME SAMPLE MULTIPLE TIMES

31-aug	BACTcontrol	4-sep	LAB analysis
8:09	280		
9:38	253	10:00	270
11:08	287	10:00	160
X	273	X	215
s	18	S	78
%RSD	6,5%	%RSD	40%

To see if there is a correlation between the results of the BACTcontrol and the culture assay, the results are plotted in Figure 28. All results from the three sampling points are used for the correlation. Figure 28 shows that the correlation between the two methods is low at Tanthof.

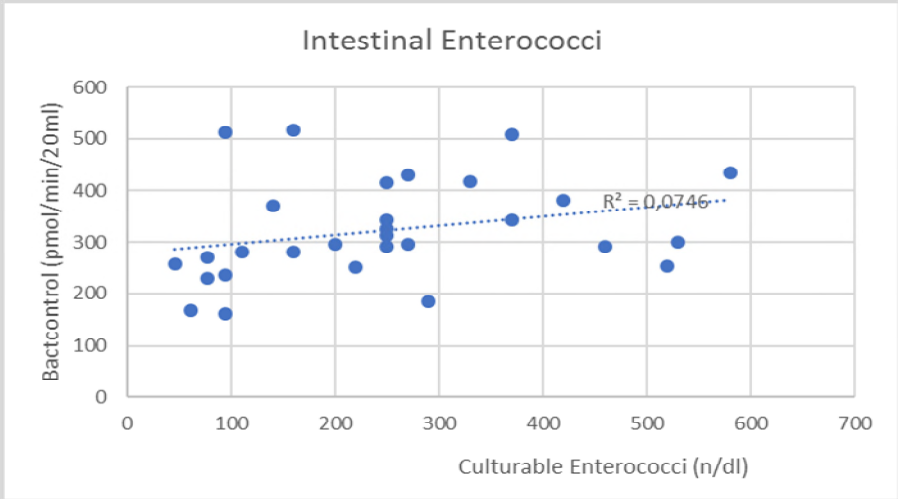

FIGURE 28. CORRELATION BETWEEN THE BACTCONTROL AND LABORATORY ANALYSES AT TANTHOF

5.3.4 Conclusions

- The BACTcontrol is easy to use. The manager of the playground could run the analyses after a short introduction;
- The BACTcontrol shows increasing bacterial activity during the day, which fits the theory and previous measurements;
- The BACTcontrol has a low standard deviation (6.5 %) compared to the culture method that was used in the laboratory (35 %);
- The results of the BACTcontrol are available within 2 hours after sampling, while the culture method takes at least two days to give a result;
- The bacterial concentration level of water playgrounds in surface water is much higher than the levels of drinking water. Therefore, a small amount of sample (20 ml) is sufficient for the BACTcontrol;
- The correlation between the results of the BACTcontrol signal and culturable enterococci is low in this experiment.

5.3.5 Recommendations

- Because of the quick availability of results the BACTcontrol can be an added value in water playgrounds. With the current method, it takes a least 2 days to get a result, so warnings about bacterial problems are late. The BACTcontrol can play a role in that;
- More research must be done to find a better correlation between the results of the conventional culture method and the results of the BACTcontrol.

5.4 Waternet WRK/WCB

The performance of the BACTcontrol in an online setting on surface water used for the production of drinking water was tested in the Amsterdam water supply system.

5.4.1 Schematic of full-scale location

At Waternet, location WRK/WCB Nieuwegein, water is abstracted from the Lekkanaal (side branch of the river Lek/Rhine) and treated with ferric chloride for removal of particles. After coagulation/sedimentation and rapid sand filtration (Figure 29), the treated water is transported to the dune area near Vogelenzang. There the water infiltrates into the dunes. The water obtained after dune infiltration is further treated to drinking water using rapid sand filters, disinfection with ozone and slow sand filtration.

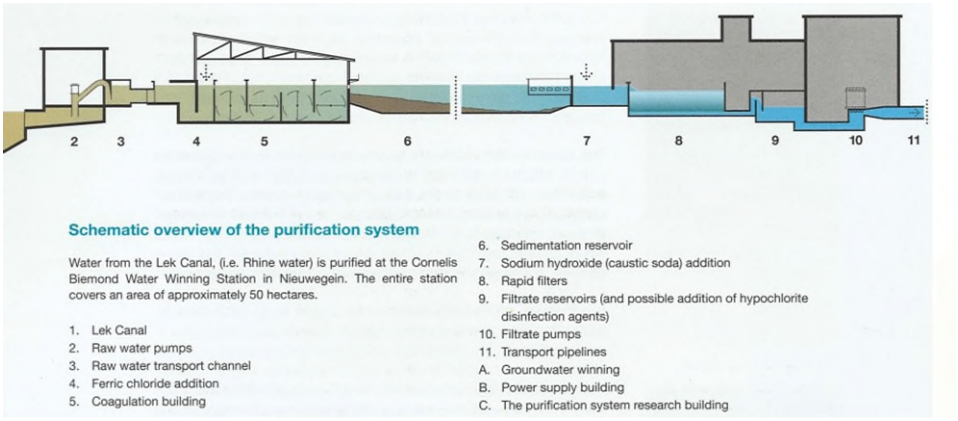

FIGURE 29. SCHEMATIC OF THE PILOT LOCATION

The BACTcontrol was installed in a cabin at the intake of the Lekkanaal-water. The Lekkanaal-water is already monitored using early warning systems, so connection to the existing network of water pipelines and digital data transport was made easily. The BACTcontrol analyzed untreated water from the Lekkanaal for enterococci. The volume of water filtrated by the BACTcontrol was set to 100 ml. A concentration of 25 mM substrate was used during the measurements. Every 2 hours and 25 minutes, a measurement of activity by enterococci present in the surface water, was obtained automatically. The test period was from the 2 - 21 April.

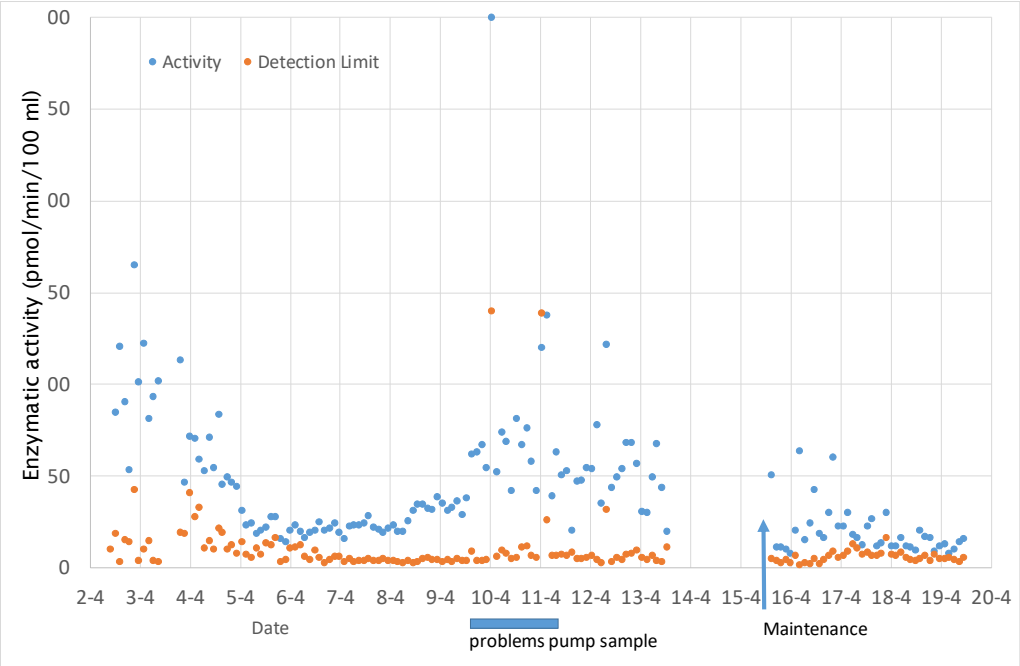

FIGURE 30. MEASUREMENTS OF ENTEROCOCCI IN LEKKANAAL-WATER AT NIEUWEGEIN WITH THE BACTCONTROL (BLUE). THE DETECTION LIMIT OF THE BACTCONTROL IS GIVEN (ORANGE).

5.4.2 Results

Figure 30 shows the BACTcontrol measurements of enterococci in Lekkanaal-water, together with the detection limit of the BACTcontrol.

In the first three days, the enzymatic activity decreased from around 100-150 pmol/min/100 ml to around 25 pmol/min/100 ml, after which the activity became stable for 4 days. On the 10th of April some problems occurred with the BACTcontrol-pump causing variation in the enzymatic activity. After maintenance on the 15th of April the enzymatic activity varied in the first 2 days. It is possible that the sensor detects smaller changes; after cleaning, the chamber and filter are clean and reflect light produced by the enzyme reaction better. The last couple of days the signal became more stable with activity around 15 pmol/min. At noon on the 19th of April, the BACTcontrol stopped caused by a blockage in a switch valve in the sample tube due to sludge particles. Maintenance by Microlan personnel was required to repair this, but due to the fact that the test period was nearing its end, this action was not performed.

On the maintenance at the 15th of April, a layer of sludge was present in the measuring chamber (Figure 31), accumulated during a 14 days period. Monitoring of the turbidity of the surface water pumped into the BACTcontrol is needed to predict the time frame when maintenance is required. Turbidity in the water sample will absorb light produced by the enzyme reaction and reduce the light that reaches the sensor, resulting in an underestimation of the enzymatic activity. So, a shorter time between maintenance could have improved the sensitivity/stability of the signal.

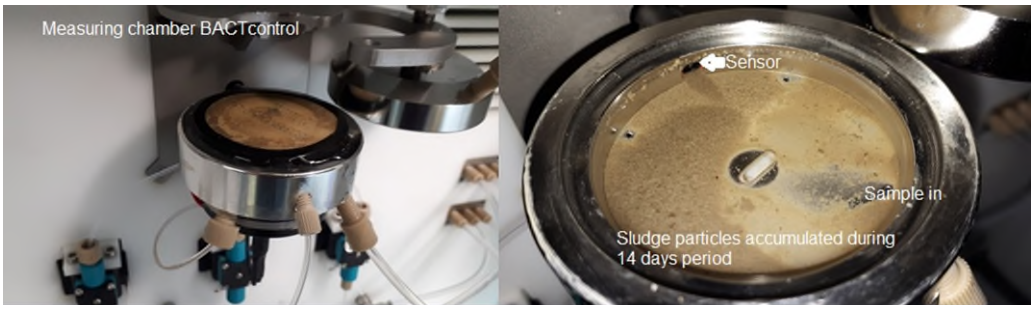

FIGURE 31. STATUS OF MEASURING CHAMBER BACTCONTROL DURING MAINTENANCE AFTER A RUN PERIOD OF 14 DAYS.

5.4.3 Conclusions

The BACTcontrol can be used as online system for detection of enterococci, as long as the turbidity of the water does not cause problems with the hardware of the BACTcontrol or with the measurement results itself. It is not known at which turbidity level measuring (surface) water will cause errors with the BACTcontrol. With Lekkanaal water, more frequent maintenance might have been needed to produce a more stable signal.

During the test period no samples for the laboratory analyses of enterococci was performed, so no correlations between different enterococci methods can be presented.

More data are needed to be able to draw conclusions regarding variation in enterococci in surface water on this particular site. A longer period is needed for this, combined with an extended monitoring program to compare the BACTcontrol results with culture methods. For online testing, better hardware is required for an optimal sample flow towards the BACTcontrol.

6 Overall discussion and conclusions

6.1 Overall evaluation of the BACTcontrol enterococci assay

The conclusions from the different activities of the study are presented in each of the chapters of this report. In this chapter, the results are discussed more generally in the light of the overall objective of the study: to further develop the enterococci assay on the BACTcontrol and evaluate the applicability for analysis of drinking water and surface water (bathing water, drinking water intake) in a laboratory and on-site setting.

In this study, the BACTcontrol was first optimized in two subsequent series of experiments in two different laboratories and the optimized assay was deployed in different laboratories and in the field. The experiences of the users with the BACTcontrol enterococci assay were collated.

There were three main research questions identified for the BACTcontrol enterococci assay:

1. The BACTcontrol is developed for deployment as an on-site, semi-continuous monitoring system, but for the enterococci assay, all tests thus far had been in a laboratory setting.
2. Within-sample reproducibility was good, but between-sample reproducibility of the relation between the BACTcontrol enzyme activity signal and the concentration of culturable enterococci was poor. This hampers the use of the BACTcontrol in drinking and bathing water quality testing.
3. The limit of detection was too high for application of the BACTcontrol for (statutory) drinking water testing.

The next paragraphs discuss and conclude about the assay optimization, user experience and the progress made in this study towards each of these research questions after the two parts of optimization of the assay and testing the BACTcontrol enterococci assay on drinking water and surface water in different laboratory and field settings.

6.2 Optimization of the BACTcontrol enterococci assay

Lysis buffer: alternative buffer solutions were evaluated; the standard buffer with 0.5% or 5% Triton X-100 and a measurement time of 60 minutes yielded the highest BACTcontrol signal, so standard buffer with 0.5% Triton X-100 was suggested as optimal.
Substrate: an alternative (cheaper) substrate manufacturer (Aeser) was evaluated, but the Sigma substrate performed better and was maintained in subsequent experiments. Alternative substrate concentrations (12.5 and 25 instead of 100 mM) were tested. In drinking water samples, without culturable enterococci, the background signal was low. There was no difference in enzymatic activity when 12.5 mM or 25 mM substrate was used in the BACTcontrol for measurements of surface waters below 50 pmol/min. Between 50 and 100 pmol/min more variability was observed. When the enzymatic activity exceeds 150 pmol/min/vol (about 1000 culturable enterococci), the use of 25 mM substrate is preferred to prevent depletion of substrate during the incubation in the reaction chamber of the BACTcontrol.

Rinsing: to improve the between-sample reproducibility, rinsing of the reaction chamber and filter with ultrapure water before the enzyme reaction was used to reduce impact of the sample matrix on the buffer and enzyme activity. This rinsing step improved the BACTcontrol signal with a factor of approximately 1.5 in the first laboratory. In the second laboratory, the extra rinsing step did not improve the enzymatic activity of the BACTcontrol. In samples of Lake IJsselmeer, high enzymatic activities were measured (higher than in samples of Afgedamde Maas) while the water contained no or very low numbers of culturable enterococci. An extra rinsing step did not improve this.
When monitoring in laboratory mode, rinsing AFTER the assay is completed is necessary to remove any remaining enzyme from the reaction chamber and prevent carry-over to subsequent samples.
Sample volume: a larger sample volume (1000 ml instead of 100 ml) improved the signal-to-detection limit ratio of the BACTcontrol enterococci assay, but the results of Afgedamde Maas did not show an improvement in the BACTcontrol signal-to-culturable enterococci relation: the BACTcontrol signal did not increase with increasing culturable enterococci concentrations. Vitens laboratory found indications for better correlation between BACTcontrol signal and culturable enterococci in 1000ml samples than in smaller volumes.

6.3 User experience

6.3.1 Operation and maintenance

The experiences recorded by users in the laboratory and in the field settings are that the BACTcontrol system is robust and easy to use, also in the field by people that have no laboratory technical background, after a short introduction. The concentration is at bathing water sites was high and required only 20 ml sample to obtain a recognizable signal in the BACTcontrol. Other points that were noted were that connection, set-up and start and stop procedures and alarms are straightforward and clear and the system responds rapidly to user interaction.
The BACTcontrol enterococci assay can be used for on-site, semi-continuous operation, as long as the turbidity and suspended solids of the test water does not cause problems with the hardware of the BACTcontrol or with the activity measurement. Turbidity and suspended solids in the test water may foul the membrane and reaction chamber and this may influence the amount of light that is received by the sensor. This will reduce the signal-to-enzyme activity ratio. More frequent cleaning is needed to prevent this. Suspended solids could block the switch-valve in the sample feed during semi-continuous operation and that prevents sample to enter the reaction chamber and thus the assay. Technical errors during semi-continuous operation were also observed in KWR2020.007. Better hardware or automated maintenance/cleaning would be needed for semi-continuous operation. It would be helpful to know the operational window for sample turbidity level and suspended solids and potentially other water quality parameters (algae?) that may distort the sampling and analysis. With Lekkanaal water, more frequent maintenance of the reaction chamber and filter might have been needed to produce a more stable signal.

After maintenance, higher sensor signals were observed that lasted for several days before returning to the background level**.** This has also been observed in the study on bacterial activity (KWR 2020.007).

Other recommended improvements were the use of larger reagent containers so refill is needed less quickly. Also, it would be beneficial to be able to pre-set different filtration volumes and time intervals for sampling.

Other points of attention from the user's perspective are that larger volumes take long to filter through the system, system cleaning is time-consuming and the enzyme activity reading produced by the blank may not return to zero after enterococci measurements.

6.3.2 Results and interpretation

Time-to-result: a key characteristic of the BACTcontrol enterococci assay is the short time-to-result: the results of the BACTcontrol are available within 2 hours after sampling, while the culture method takes one to two days.
Background signal (noise): the background signal of the BACTcontrol when monitoring drinking water in an on-line setting is low (0 – 4 pmol/min).
Linearity: the BACTcontrol enterococci assay is linear when different volumes of the same sample are tested.
Reproducibility: in the same (bathing water) sample, the BACTcontrol has a low standard deviation (6.5 %) compared to the culture method (35 %). Between sample reproducibility is low and makes it difficult to interpret the BACTcontrol activity reading to culturable enterococci concentrations and existing water quality standards and hence to communicate with process operators or others about the significance of the results.

6.4 Between-sample reproducibility

Despite the optimization steps, each of the users of the BACTcontrol assay found a low correlation between the BACTcontrol signal and the number of culturable enterococci between samples. KWR observed this for the samples of the Lekkanaal and de Waal, Het Waterlaboratorium for samples of IJsselmeer and Afgedamde Maas, Vitens laboratory for the Klaarbeek at Epe and Hoogheemraadschap Delfland at the bathing waters at Buytendelft and Tanthof. The enterolert culture assay of the IJsselmeer samples. That uses the glucosidase activity in combination with culture, showed low concentrations of culturable enterococci where the BACTcontrol yielded a high signal. The fact that within-sample reproducibility is high implies that the BACTcontrol enterococci assay can be reproducible, so the low between-sample reproducibility is an indication of matrix effects. Matrix effects could be the presence of compounds in the matrix that hinder the action of the buffer to provide access of the substrate into the enterococci cells or hinder the enzyme activity or hinder the amount of emission light reaching the MU fluorophore and fluorescent light reaching the sensor. In semi-continuous operation mode, users observed fouling of the membrane and reaction chamber after 14 days of operation on Lekkanaal water. The amount of fouling was not studied in the other settings, but will be less in the situations where the BACTcontrol was run in batch mode. Rinsing the reaction chamber and ceramic filter with ultrapure water to remove remaining sample matrix did not consistently improve the BACTcontrol signal or correlation between BACTcontrol signal and culturable enterococci.

Matrix effects can differ between sampling sites and also between samples at different place or time at the same sampling site. If the matrix effect would be site-specific, the BACTcontrol to culturable enterococci ratio would be relatively consistent at each site and could differ between sites. That would imply that the BACTcontrol could be used after site specific 'calibration' against culturable enterococci concentration. However, if the variability in the BACTcontrol-culture ratio also occurs between samples at the same

site, this would make the interpretation of the BACTcontrol signal very difficult. Although not systematically investigated in this study, comparison of the BACTcontrol signal to culturable enterococci at the different surface water sites tested suggests that the between-sample variability is more than a between-site variability. The correlation between BACTcontrol and culture was low (R^2<0.1) for the bathing water sites Buytendelft and Tanthof. For the Klaarbeek at Epe, the correlation was mediocre (R^2=0.52). Therefore, continuous monitoring at sites like Klaarbeek could be an opportunity for successful deployment of the BACTcontrol enterococci assay, provided the technical issues (see 6.3) are largely resolved. Here, the best correlation was found with a filtration volume of 1000ml.

6.5 Limit-of-detection in drinking water samples

The background signal of the BACTcontrol in all drinking water samples (without culturable enterococci) tested is low, both in laboratory mode and in semi-continuous mode. The background signal did differ considerably between drinking water from different sites, with relatively high levels in Nieuwegein tap water and low levels at Vitens laboratory tap water, where 1000ml samples were tested.

No direct comparison of BACTcontrol signal with culturable enterococci in drinking water samples is possible, because none of the drinking water samples contained enterococci. The surface water data of the BACTcontrol versus culturable enterococci showed low between-sample reproducibility and suggest that relatively high numbers of culturable, environmental enterococci need to be present in the BACTcontrol to produce a clear signal. However, the observation in the Tanthof bathing water that high culturable enterococci were not paralleled with a high BACTcontrol signal suggests that the BACTcontrol does not consistently produce a clear signal when culturable enterococci are high.

6.6 Event detection with the BACTcontrol

During the operation in semi-continuous mode at the bathing sites, the BACTcontrol signal varied parallel to the concentration of culturable enterococci on Aug 31 and Sep 2 at 4pm in the Tanthof. The observations of the local bathing water manager that visitor counts increased as the day progressed fits to the BACTcontrol measurements. The culture method does not show that pattern. On Sep 2 at 8am and Sep 5 at 8 and 10 am, the culture count was increased, but the BACTcontrol did not show a clearly increased signal. On Sep 1, the BACTcontrol signal increased, while culturable enterococci did not show an increase. Based on these results from Tanthof, it seems that the BACTcontrol enterococci assay is detecting changes in faecal contamination of bathing water in some instances but not in others. More data are needed to evaluate this at this and other locations.

6.7 Recommendations

The research within this TKI project showed that the BACTcontrol enterococci assay is easy and can be run at laboratory and semi-continuous mode, but also highlighted technical limitations that hamper semi-continuous operation and low maintenance. Several recommendations (see 6.3) were made for improvements of the BACTcontrol system operation and maintenance based on the users' experience.

Looking at the findings of this study, the BACTcontrol enterococci assay does not seem to have a limit-of-detection that is low enough for assaying drinking water at a level of 1 per 100 ml, even after optimization and increasing the sample volume to 1000ml. Drinking waters (without culturable enterococci) produce a low background signal in

the BACTcontrol, at the Vitens laboratory tap water this was very low. Such low background tap waters would be a good site to evaluate the limit of detection in drinking water further. It is not clear what determines the difference in the background level between drinking water sites. Analyzing the cause of the differences could provide information about what water quality parameters impact the BACTcontrol enterococci assay signal (and possibly other signals of the BACTcontrol, see KWR2020.007).

The between-sample differences in the BACTcontrol enterococci assay are still not understood and hinder successful application of the BACTcontrol for the enterococci assay. Rinsing did (not consistently) improve the BACTcontrol signal, as did cleaning of the reaction chamber and ceramic filter, suggesting that matrix effects do reduce the BACTcontrol signal. Also the carry-over between subsequent assays without proper cleaning after the assay occurred. A more rigorous cleaning and rinsing protocol of the reaction chamber after each sample may increase the BACTcontrol signal more consistently.

Successful deployment of the BACTcontrol for semi-continuous monitoring is hampered by the low between-sample reproducibility, as indicated by the two bathing site tests. The only site with a correlation between BACTcontrol signal and culturable enterococci was with the Klaarbeek at Epe, tested in offline mode in the Vitens laboratory by taking samples at the site, transport them to the lab and analyze them with the BACTcontrol and conventional culture assay. This correlation could have been impacted by the presence of a number of the same samples with different sample volumes in this data-set, but testing the BACTcontrol in semi-continuous mode at this site (again, provided that the technical issues (see 6.3) are resolved, could indicate whether application of the BACTcontrol enterococci assay is feasible in this surface water.

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