Chapter 2

In situ monitoring of fecal bacteria at the Seine Valenton WWTP using ALERT rapid microbiology instrumentation (Fluidion®)

© SIAAP

2.1 INTRODUCTION

Identifying the presence of fecal indicator bacteria and measuring their concentration is a critical aspect of water quality monitoring, exerting a direct impact on safety aspects related to water usage: a safe drinking water supply,

doi: 10.2166/9781789062106_0064

recreational and competitive uses (swimming, boating, aquatic sports), agricultural use (irrigation), as well as aquaculture operations. Exposure to fecal pathogens through contact with contaminated water constitutes a major health risk, causing a wide variety of illnesses and infections with potentially fatal consequences, and moreover is recognized by the World Health Organization as such a health risk (WHO, 2003). Microbiological pollution with human and animal waste pathogens can be caused, in urban areas, by bacteria from wastewater plant effluent. This effect can be amplified during heavy rain episodes by combined sewer overflow (CSO) phenomena and, in some cases, by illegal discharges, boat sewage and faulty connections to the sewage infrastructure. In rural settings, agricultural runoff (e.g., from livestock operations) as well as the natural presence of birds and other warm-blooded animals are often responsible for microbiological pollution.

Monitoring the microbiological quality of drinking water and surface water in sensitive areas (i.e., where direct human contact or food chain contamination can create a health risk) is mandatory throughout the industrialized world and typically performed by measuring concentrations of viable and cultivable fecal indicator bacteria (FIB). These bacteria are generally not considered to be illness vectors by themselves, but rather provide an accurate tracer or proxy (Prüss, 1998). Depending on the country, the choice of specific FIB used for monitoring may vary: *E. coli* is preferred for general public health protection in fresh water (Edberg *et al.*, 2000); intestinal enterococci are more prevalently monitored in seawater environments, where they often complement or even replace *E. coli* measurements; and Thermotolerant Coliforms and Generic Total Coliforms are used in certain parts of the world, or for specific water matrices.

Typical standard regulatory FIB monitoring methods tend to follow one of two approaches. The more common approach is the most probable number (MPN) method, which employs either micro-assays (e.g., 96-well microplates (ISO 9308-3 1998), as the method used in many European countries) or larger-scale assays (e.g., Quanti-Tray 2000 (Quanti-Tray is a trademark or registered trademark of IDEXX Laboratories, Inc. or its affiliates in the United States and/or other countries) (ISO 9308-2 2012), now widespread in the United States, or multiple-tube fermentation (EPA 9131), now mostly obsolete). The second approach consists of membrane filtration followed by plating on a chromogenic Agar medium; it is generally reserved for low-concentration samples, such as in drinking water studies (ISO 9308-1 2014) These standard regulatory methods are quite often slow, with a typical time-to-response (TTR) being between 24 and 72 hours when taking into account all sampling, transport, storage and quantification protocol steps. Many situations require significantly faster early-warning systems in order to impose access closures or adapt operations accordingly. This need is particularly important wherever high risks of human contact with contaminated water are involved, such as when discharging upstream of a sensitive area (e.g., drinking water intake, recreational activities,

irrigation of fresh produce, various forms of aquaculture). A strong need exists in the water and environmental engineering industry for rapid measurement devices capable of performing a reliable *in situ* quantification of the viable indicator bacterial load.

Previously reported online quantification techniques have generally focused on *E. coli*, while some have been applied to surface water quality monitoring (Lopez-Roldan *et al.*, 2013; Noble & Weisberg, 2005). Attempted enumeration methods range from simple light-scattering measurements to direct color and fluorescence measurements (Baker *et al.*, 2015), including complex molecular techniques. Certain methods, such as Reverse-Transcription Quantitative PCR (Bergeron *et al.*, 2011) and direct measurement of enzymatic activity (Baudart *et al.*, 2009; Briciu-Burghina *et al.*, 2015; Burnet *et al.*, 2019; Heery *et al.*, 2016; Wildeboer *et al.*, 2010), can provide initial results very quickly, in as little as 4 hours; however, such methods are prone to counting both cultivable and non-cultivable bacteria and are easily interfered by other types of microorganisms or by free enzymes present in the sample. This observation is particularly true of enzymatic techniques without a selective growth step; moreover, it becomes highly relevant whenever such methods are performed in a wastewater environment, where disinfection techniques may deactivate, but not kill, bacteria and where high background levels of free enzymes exist. For all these reasons listed, such methods do not generally provide a very robust correlation with regulatory measurements focusing specifically on viable, cultivable cells (Burnet *et al.*, 2019). Fluorescent *in situ* hybridization has been applied to bacterial detection as well (Baudart & Lebaron, 2010), although it remains limited to laboratory usage due to significant protocol complexity. Defined Substrate Technology (DST), combining a selective growth medium for the bacteria of interest with enzyme substrates linked to specific chromogens and/or fluorogens produced by bacterial metabolism, stands out as a reliable detection technique. Several approved quantification methods combining DST assays with MPN techniques also exist and have been standardized (ISO 9308-2, ISO 9308-3).

The Fluidion® ALERT line of instrumentation for monitoring microbiological contamination is a novel technology that utilizes a modified real-time DST method for bacterial enumeration. Fluidion® ALERT technology allows for a fully-automated *in situ* quantification of viable and cultivable generic *E. coli* and total coliforms or, alternatively, of the intestinal enterococci concentration or fecal coliform concentrations in both fresh water and seawater environments. The TTR ranges between 2 and 12 hours (shorter response times correspond to higher concentrations), and the limit of detection corresponds to one target bacterium in the 25-mL sample volume (i.e., a limit of detection of four bacteria/100 mL in fresh water, which if applied for specific protocols would need to be multiplied by the pre-dilution factor). An upper measurement range of 5×105 bacteria/100 mL is obtained with no need for serial sample dilution, thus covering in a single measurement over five orders of magnitude in concentration. The detailed

calibration and metrological validation results for fresh surface water *E. coli* enumeration have been published elsewhere (Angelescu *et al.*, 2018a). The fact that no sample transport, conditioning or preparation is necessary yields certain logistical advantages and eliminates the risk of sample degradation and human error present in current techniques, while providing rapid and reliable information on water quality to enable effective decision-making. ALERT technology has been employed in numerous applications worldwide, ranging from seawater monitoring to the *in situ* environmental monitoring of highly-polluted streams (Angelescu *et al.*, 2018b), including source-identification investigations performed by regulatory agencies (Cronin *et al.*, 2018; Loewenthal *et al.*, 2018) and non-profit organizations (Angelescu & Saison, 2020) and implementation on various platforms, notably in conjunction with remotely-controlled aquatic drones (Angelescu & Hausot, 2019).

Since 2017, Fluidion® ALERT technology has been deployed at multiple sites along the Seine River in Paris to perform high-frequency rapid *E. coli* quantification in river water, thus complementing long-term monitoring efforts pursued by multiple entities involved in the short water cycle (City of Paris, SIAAP (SIAAP: *Syndicat Interdépartemental d'Assainissement de l'Agglomération Parisienne* is the Greater Paris Sanitation Authority) and neighboring departments) aimed at establishing environmental baselines and quantifying the impact of various mitigation actions adopted as early as 2000. Initial mitigation efforts, aimed at limiting phosphorus and nitrogen influx and reducing sewer overflow during rainstorm events, have resulted in continuous improvements to the microbiological quality of the Seine River over the past few decades (Rocher & Azimi, 2016).

Recent years have witnessed strong renewed interest in natural bathing sites, not only along the coast but also in urban environments, by both public authorities, through increasingly considering urban rivers as recreational resources, and the public, through various grass-roots actions (Ziegler, 2019). Competitive sports are, in turn, becoming increasingly cognizant of the urban environment. For example, the successful 2024 Olympic bid by the City of Paris includes the provision that certain aquatic events will take place in the Seine River. Mitigation efforts by the oversight entities therefore need to address not only wet-weather but also dry-weather pollution in urban environments; the reduction of bacteria in treated wastewater requires implementing some form of wastewater treatment plant (WWTP) effluent disinfection. Assessing and optimizing the effectiveness of such a disinfection method implies the ability to monitor FIB concentrations throughout the wastewater treatment process.

The present study describes a new wastewater measurement protocol implemented using Fluidion® ALERT technology. The calibration and validation procedures were carried out with a wide range of wastewater samples obtained from typical modern wastewater plants. ALERT results were compared side-by-side with analyses performed by an approved third-party laboratory using

the MPN microplate standard method (ISO 9308-3 1998). Samples were included from all the relevant treatment stages: primary clarification, decarbonation, nitrification, denitrification, and tertiary activated carbon. Moreover, results were presented from a full-scale WWTP chemical disinfection pilot, where Fluidion® ALERT technology was deployed operationally to provide high-frequency monitoring of the *E. coli* concentration in both untreated and disinfected effluent, thereby measuring the abatement factors under several different operating conditions.

2.2 EXPERIMENTAL DESCRIPTION

2.2.1 Description of the ALERT technology

Fluidion® ALERT technology is based on a modified real-time DST method, as implemented in automated instruments capable of performing, directly in the field, the complete protocol for bacterial quantification. ALERT instruments can automate the full range of operations: sampling, reagent mixing, incubation, real-time multispectral optical analysis (absorbance/fluorescence), turbidity correction, signal analysis, bacterial quantification, wireless data transmission, and automatic generation of notifications. ALERT technology can be deployed in multiple configurations: ALERT System for performing automated *in situ* bacterial enumeration to obtain time-series data at a target location; and ALERT LAB as a portable device for the rapid mapping of bacterial contamination at multiple sites or as a bench-top device for rapid laboratory analysis (Figure 29). Both ALERT System and ALERT LAB devices have been used in the present study.

The bioreagent used in ALERT instruments contains a mixture of a selective growth medium and 4-methylumbelliferyl-β-D-glucuronide (MUG), which can be hydrolyzed into fluorescent 4-methylumbelliferyl (MUF) by the β-glucuronidase enzyme present in *E. coli* bacteria (note: MUG is the standard substrate used in approved *E. coli* testing methods). The bioreagent used also contains ortho-nitrophenyl-β-galactoside (ONPG), another bacterial indicator metabolized by all types of coliforms in the sample and transformed into ortho-nitrophenol (ONP), resulting in the development of yellow coloration. During the selective culture step (involving incubation at 37.0°C), bacterial metabolism progressively transforms MUG into MUF, in generating a broad fluorescence when excited at 385 nm, with emission peaking at around 460 nm. The growth of non-target organisms is not promoted during this selective culture step, which makes the method highly selective to culturable *E. coli*, unlike rapid tests based solely on enzymatic activity without culture.

All ALERT instruments contain multiple individual bioreactors (six for the portable ALERT LAB, seven for the *in situ* ALERT system), each capable of independently incubating a sample and performing optical measurements with an optical sensor ring. This sensor ring contains three LEDs, arranged to excite MUF fluorescence (385-nm excitation), measure ONP absorbance (430 nm) and

compensate for sample turbidity (610 nm), as well as a photodiode coupled to a low-pass optical filter that blocks the UV excitation light. The fluorescence signal is measured at periodic intervals (every 5 min), with data transmitted in real time through the mobile phone network to a remote cloud-based data server. The resulting curve (Figure 30) consists of an initial plateau, followed by a sharp increase in fluorescence starting a few hours into the measurement. The curve is automatically analyzed by the data server to establish the fluorescence detection time, which is then used, after applying a specific calibration, to calculate the number of bacteria present in the original sample.

2.2.2 Laboratory reference method

The reference method employed for all side-by-side comparisons is the miniaturized MPN technique using a 96-well microplate (ISO 9308-3 1998). This method consists of performing multiple dilutions of the sample to be analyzed, according to a protocol that depends on the expected degree of pollution; the 96 wells, pre-loaded with the *E. coli* reagent, are then inoculated with the various sample dilutions and incubated. The number of positive wells upon each dilution is counted, and an MPN table is used to determine the bacterial concentration and corresponding 95% confidence interval. The dilution series used in this study has been adapted to wastewater analysis: six dilutions were performed (1/2, 1/20, 1/200, ½,000, 1/20,000 and 1/200,000); the resulting measurement range extended from 60 to 6.7×10^8 MPN/100 mL.

2.2.3 Study design

The first part of this study consisted of analyzing a number of samples side-by-side using both Fluidion®ALERT technology and the laboratory reference method, in order to obtain and then validate a calibration function corresponding to wastewater measurements. Samples were collected from the 'Seine Centre' WWTP located in Colombes, which treats 240,000 m^3 of wastewater daily, as generated by 900,000 residents of the Greater Paris Region. The plant's treatment process is typical of a modern WWTP: a pretreatment stage (screening, grit and oil/grease removal) is followed by primary settling or clarification (organic sludge and chemical phosphorus removal) and biofiltration treatment (decarbonation, nitrification, denitrification). After these stages, the final effluent is released into the Seine River. The Seine Centre WWTP is also equipped with a tertiary activated carbon filtration pilot for a fraction of the effluent; however, no specific disinfection process is currently applied. The treatment process is effective in removing carbon, nitrogen and phosphorus, with global efficiencies of between 70 and 94%. The nitrification step also results in significant FIB removal, with four orders-of-magnitude *E. coli* abatement (from 10^7 MPN/100 mL after the clarification stage to 10^4 MPN/100 mL in the final effluent);

applying an additional tertiary activated carbon treatment was shown to further reduce the bacterial load to below laboratory detection limits (Mailler, 2015).

To ensure a broad range of FIB concentrations, a total of 125 samples were collected at the end of five different stages of the treatment process: primary clarification (26 samples), decarbonation (24 samples), nitrification (24 samples), denitrification or regular effluent (32 samples), and tertiary activated carbon treatment or treated effluent (19 samples). The samples were collected in sterile polypropylene containers, homogenized and then separated into two parts. The first part was analyzed immediately using the ALERT LAB instrument, while the second was treated with sodium thiosulfate (20 mg/L) and sent for same-day delivery and analysis at an accredited laboratory for the reference *E. coli* analysis.

During the initial phase, 49 samples were analyzed using the standard surface water protocol and calibration that had previously been developed for river water (Angelescu *et al.*, 2018a), in order to test its applicability to wastewater (test phase). Following several initial measurement anomalies (described below), it was decided to adapt the protocol by adding a sample dilution step (1/4 dilution in deionized water). The revised protocol, applied to 41 samples, eliminated the observed anomalies and resulted in establishing a new calibration specifically adapted to wastewater analysis (calibration phase). This new calibration was then validated on 35 additional samples (validation phase).

The second part of this study consisted of operationally deploying ALERT instrumentation at the Valenton WWTP during the full-scale disinfection trials. PFA was injected into the plant's effluent stream, which then traveled through a subsurface passage to the discharge point in the Seine River (Figure 28). The effluent transit time between the injection and discharge points was approximately 10 min, hence representing the disinfection product contact time. Two ALERT system instruments were used to monitor *in situ* the bacterial concentration in near real-time of both the pre-disinfection (at the plant location, Figure 28 – n°1) and post-disinfection (at the effluent discharge location, Figure 28 – n°2) to determine *E. coli* abatement during successive 24-hour periods. Another three samples were collected manually at both locations at the beginning and end of the 24-hour period, for purposes of laboratory verification.

All data from the ALERT instrumentation were sent in real time to a central data server, which ran the detection algorithm and output the resulting *E. coli* quantification. The automated ALERT data and laboratory reference data were then centralized and analyzed using Microsoft Excel, as well as custom data processing scripts written in Python language.

2.3 RESULTS OF A SIDE-BY-SIDE LABORATORY COMPARISON

The initial test phase of the study involved analyzing 49 samples from all treatment stages using the previously developed surface water protocol (Angelescu *et al.*,

2018a). On the more heavily concentrated samples (primary clarification and decarbonation stages), the fluorescence signal curves often showed aberrant behavior, with the fluorescence signal increasing linearly immediately after sample collection and without the typical plateau observed in Figure 30. This observation is typical of samples that contain very high concentrations of free MUF enzymes not contained within *E. coli* cells, even before the bacterial metabolism starts its production. Such samples can lead to major quantification errors; therefore, it was decided to modify the protocol by applying a 1/4 dilution step to the wastewater samples, thus decreasing the initial enzyme concentrations and leading to the recovery of normal signal response curves. This revised WWTP protocol has been employed throughout the remainder of the study.

During the calibration phase, 41 samples from all treatment stages were analyzed side-by-side by both ALERT technology and the laboratory reference method. Detection was performed automatically; fluorescence detection times t_{fluo} for the samples analyzed herein ranging from 4.25 to 8.50 hours, with shorter times corresponding to higher concentrations. The laboratory concentration C was plotted against t_{fluo} in a log-linear graph; a linear correlation was observed, yet the slope of the previous surface water calibration was found to be different.

A new calibration specific to wastewater samples (WWTP calibration) was therefore established between C (measured in *E. coli*/100 mL) and t_{fluo} (measured in hours), that is,: $\log_{10}(C) = a \times t_{fluo} + b$, with a and b being derived by least-squares regression (Figure 31, left). The new calibration (dashed line), as well as the previous calibration (solid gray line), are also shown on the plot.

During the validation phase, 35 additional samples were analyzed using the newly developed WWTP protocol/calibration; results were compared against the laboratory reference method.

Figure 31 (right) displays all data points from the study, separated by their respective phase (test, calibration, validation), with all points corresponding to aberrant curves from the test phase being removed. The error bars correspond to the statistical 95% confidence intervals of the MPN measurements, as communicated by the laboratory. It can be confirmed that all results closely match the calibration curve, thus indicating good correspondence over more than five orders of magnitude, with a global Pearson correlation coefficient at 88.9%, as calculated on the actual measurement values and not on the base-10 logarithm values. 46% of the ALERT results differ by less than a factor of two from the laboratory reference measurement, while 93% differ by less than a factor of five.

The histogram shown in the inset in Figure 31 is symmetrical and closely approximated by a log-normal distribution centered at 0 (red line), implying that the Fluidion® ALERT measurement method does not introduce any systematic errors after calibration. The mean standard deviation of the $\log_{10}$ difference distribution over the full measurement range is: $\sigma_{ALERT-LAB} = 0.387$ $\log_{10}$ units, which combines the uncertainty of the laboratory reference method with that of the ALERT WWTP method (acting as independent normal variables):

$\sigma_{ALERT-LAB} = \sqrt{\sigma_{ALERT}^2 + \sigma_{LAB}^2}$. The mean statistical standard deviation of the reference method over the full range of concentrations can be calculated from the 95% confidence intervals provided by the laboratory, thus yielding: CI = 0.304 $\log_{10}$ units. The mean statistical laboratory standard deviation is then obtained as: $\sigma_{LAB} = CI/1.96 = 0.156$ $\log_{10}$ units (assuming a normal distribution). We can therefore infer an upper limit for the mean standard deviation of the ALERT WWTP measurement: $\sigma_{ALERT} = \sqrt{\sigma_{ALERT-LAB}^2 - \sigma_{LAB}^2} = 0.354$ $\log_{10}$ units. Let us note that this is likely to be an overestimation of the actual measurement uncertainty since the actual laboratory standard deviation is significantly higher than the purely statistical confidence interval related to the MPN method, which does account for uncertainties due to sample degradation in transport or to homogenization errors. As estimated elsewhere (Angelescu *et al.*, 2018a), the actual laboratory uncertainty obtained by comparing measurement duplicates may be significantly higher, which would lead to a lower value for σ_{ALERT}. Unfortunately, duplicate measurements were not available in the context of this study.

It is also important to recognize that a notable difference exists between the ALERT method and MPN methods, in that the former measures the actual activity of all viable and culturable bacteria in the sample volume (without requiring sample homogenization), whereas the latter divides the sample volume into a finite number of aliquots (microplate wells), in assessing the number of aliquots registering positive for the bacteria of interest. While a fully homogenized sample should, in theory, lead to equivalent results using either method, in reality the effectiveness of laboratory homogenization may depend on the nature of the contamination and can be imperfect, thus leading to the presence of particles aggregating large number of bacteria in some aliquots, yet which are still counted as single positives. ALERT technology, on the other hand, measures the full viable and culturable bacterial load, which may be a more relevant parameter for the potential health impacts of contaminated water. As shown in this study, even though ALERT measurements correlate very well with laboratory MPN results in the vast majority of cases, differences may appear for samples containing a large fraction of bacteria aggregated on particles, which may be underestimated by the MPN method.

Figure 32 below further displays the samples analyzed during the WWTP protocol calibration and validation phases, color-coded by the treatment stage where each sample was collected (as indicated in the legend). The right-hand panels focus on results obtained per individual treatment stage. It can be observed that the newly developed WWTP protocol for ALERT technology is able to accurately measure all the types of wastewater samples tested, regardless of their origin or concentration.

One of the ultimate objectives of this work is to develop a rapid method for assessing the impact of WWTP effluent disposal on surface water quality in view of recreational activities. The previous analysis was therefore reinforced by

applying standardized guidelines (US EPA 2014) for confirming the applicability of site-specific novel *E. coli* measurement methods in assessing recreational water quality. The US EPA site-specific criteria rely on calculating the association between two methods, by applying acceptability thresholds to both the index of agreement *IA* and R^2 value, calculated using the base-10 logarithm of the measurement values from each method. The US EPA criteria consider that agreement between the two methods is sufficient when $IA \geq 0.7$; if this condition is not satisfied, then the R^2 value allows estimating how well the two methods are correlated. If $R^2 > 0.6$, then the alternative method can be applied, but with new numerical limits that need to be derived. When applied to all data obtained using the WWTP protocol, we calculate $IA = 0.98$ and $R^2 = 0.92$, which lie above the thresholds required by the standardized guidelines (0.7 and 0.6), thus indicating excellent agreement between the two methods as per the US EPA site-specific criteria for recreational water quality.

2.4 COMPARISON OF *IN SITU* RESULTS ON WWTP EFFLUENT DISINFECTION

Results from the second part of the study, consisting of ALERT instrumentation deployment at the Valenton WWTP (France) within an operational environment, have enabled measuring the *in situ* efficacy in a full-scale chemical disinfection pilot using PFA. Seven measurements were performed by each of the pre- and post-disinfection ALERT systems during the disinfectant injection experiment, at 4-hour intervals. A 10-minute delay was introduced between the start of the pre- and post-disinfection measurements, in order to account for the transit time of the effluent between the two installation sites. Three samples were also collected by hand from each location, at the beginning and end of the 24-hour measurement period, and then sent to a certified laboratory to measure the *E. coli* content using the reference method. The results are presented graphically as a time series (Figure 33) and summarized in Table 12.

The ALERT and laboratory reference measurements produced similar measured concentrations both pre- and post-disinfection, with abatement factors of 3.4 and 2.7 $\log_{10}$ units as measured by the ALERT system and laboratory, respectively.

Key points

- ALERT technology was tested on various wastewater matrices and a new calibration was developed and validated in side-by-side comparisons with the laboratory reference method.
- The ALERT method was shown to accurately determine *E. coli* concentrations over more than five orders of magnitude, corresponding to all wastewater treatment stages: primary clarification, decarbonation,

nitrification, denitrification, and tertiary treatment. The ALERT WWTP protocol required only a single initial dilution at 1/4 (as compared to six dilutions needed for the reference method).

- Total measurement time for the samples analyzed in this study ranged from 4.25 to 8.50 hours, with shorter times corresponding to higher bacterial concentrations (as compared to the 24–72 hours required to obtain the reference method results).
- Following laboratory validation, ALERT technology was integrated into an industrial wastewater chemical disinfection pilot to monitor the operational performance and determine the abatement factor obtained through different disinfectant exposures, both *in situ* and in near real-time. Significant improvements in reliability and logistical complexity were demonstrated through deployment of automatic ALERT technology, leading to faster results while fully eliminating the need for laboratory analysis and avoiding sample degradation due to transportation or human error.