DEMOWARE

Deliverable D2.5
Applicability of flow cytometry and qPCR methods to assess and control microbial contamination

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Abstract	This report presents the results of Task 2.5 for the WP2 of DEMOWARE in relation to the applicability of flow cytometry and qPCR methods to assess and control microbial contamination within water reuse applications. Application of the devices and technologies are applied to a water recycling plant and associated non-potable distribution network, irrigation scheme and wastewater treatment plants with varying disinfection steps. Findings evaluate the applicability of the stated techniques including limitations and aims to provide assistance to water operators who operate/plan to operate a water reuse scheme on the application of these techniques to monitor treatment efficacy, performance and inform maintenance activities.

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Executive Summary

Water utilities routinely monitor microbiological parameters through time consuming cultivation methods (i.e. plate counts) at a frequency that would not provide early warning of a contamination event. Adoption of a similar sampling and monitoring programme for water recycling schemes are viewed less favourably through the inherent risk associated to the wide range of potential impacts of contaminants within the source wastewater. As such, more effective control measures are favoured to enable early identification of contamination thereby reducing the risk associated with water reuse applications.

Recently, new fluorescence-based technologies offer significant advantages and overcome the limitation of conventional plate count methods. These alternative and more rapid diagnostic devices and techniques characterise a microbiological community and the efficacy of the disinfection process independent of cultivability within a matter of minutes to hours, thereby providing early warning of a contamination event. Furthermore, these alternative devices can be utilised on site by trained personnel thereby eliminating the requirement for the sample to be couriered to a specialised lab for analysis.

This report aims to assess examples of these new devices and techniques in the evaluation of the effectiveness of multi-barrier treatment water reuse schemes and disinfection technologies. In doing so, the report aims to assess:

- The applicability of the devices and techniques for microbial contamination within water reuse applications including limitations
- Effectiveness of treatment processes based on surrogate parameters and relation to maintenance regimes

The systems selected for evaluation of applicability include flow cytometry (FCM) and a molecular indicator system quantitative real time polymerase chain reaction (qPCR).

The application of FCM to monitor multi-barrier treatment process was found to achieve consistent results which indicated a change in water quality following physical treatment, disinfection and storage periods. The results generated provided a baseline profile for the treatment process within each scheme thereby enabling determination of performance deterioration and maintenance requirements through deviations within the baseline values. Results acquired through FCM could be associated to surrogate parameters including suspended solids (irrigation application) and disinfectant residual (non-potable network), enabling the determination of a bespoke disinfectant residual to limit cell viability and regrowth during distribution and assist with maintenance activities. Limitations in the technique were concluded regarding the limit of detection, with highly concentrated samples requiring pre-sample preparation and whilst dilute samples contributed to biased estimations of efficacy. In addition, the technique was found to be most appropriate in the determination of an intact/alive cell concentrations following chemical disinfection, due to the method of cell compromisation targeted through the double staining methodology (i.e. cell membrane damage), in comparison to non-chemical processes such as ozone.

Unlike FCM, qPCR was possible for highly enriched bio-film samples and could detect non-cultivable yet hygienically relevant microorganisms. Furthermore, sample preparation could be standardized via existing DNA extraction protocols and in combination with a DNA stain can distinguish between viable and membrane-compromised cells. However, the data to date is not sufficient to draw final conclusions regarding specific treatment processes and maintenance regimes similar to FCM with future results enabling the possibility to understand the impact of specific treatment on the population dynamics of different wastewater treatment plants, and identification of critical and hygienically relevant organisms.

Abbreviations

AOC	Assimilable organic carbon
BlueBiolabs	Blue Biolabs GMBH
CFU	Colony Forming units
Cl	Chlorine
DOC	Dissolved organic carbon
ds	Double-stranded
DST®	Defined Substrate Technology®
FCM	Flow cytometry
FSC	Forward scatter
GAC	Granular activated carbon
GW	Well water
HNA	High nucleic acid
HPC	Heterotrophic plate count
ICC	Intact cell concentration
IRSA-CNR	Consiglio Nazionale delle Ricerche
LNA	Low nucleic acid
LRV	Log removal values
MBR	Membrane bioreactor
MLGA	Membrane Lactose Glucuronide Agar
MLSS	Mixed liquor suspended solids
MPC	Media Press Centre
MPN	Most probable number
MUG	Fluorogen 4-methyl-umbelliferyl glucuronide
NTU	Nephelometric turbidity units
OFWRP	Old Ford Water Recycling Plant
ONPG	O-nitrophenyl-β-D-galapyranoside
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDT	Pressure decay testing
PE	Population equivalence
PI	Propidium iodide
PMA	Propidium monoazide
PMT	Photomultiplier detectors
PPi	Inorganic pyrophosphate
QEOP	Queen Elizabeth Olympic Park

qPCR	Quantitative real time polymerase chain reaction
RAS	Returned activated sludge
RAS	Returned activated sludge
SG I	SYBR Green I
SRP	Soluble reactive phosphorus
SSC	Side scatter
SW	Secondary effluent
TCC	Total cell concentration
TN	Total nitrogen
TOT	Complete treatment train
TSS	Total suspended solids
TW	Tertiary effluent
TWUL	Thames Water Utilities Limited
UCRAN	Cranfield University
UF	Ultrafiltration

1 Introduction

Water utilities routinely monitor microbiological parameters through periodic grab sampling programs at a frequency that would not provide early warning of a contamination event. Adoption of a similar sampling and monitoring programme for water recycling schemes are viewed less favourably through the inherent risk associated to the wide range of potential impacts of contaminants within the source wastewater. As such, more effective control measures are favoured to enable early identification of contamination thereby reducing the risk associated with water reuse applications.

By treating the effluent with chemical disinfectants many (preferably most) of the contained bacteria are damaged by oxidative reactions and hence rendered nonviable. Conventionally, time-consuming (up to several days) cultivation methods are used to assess disinfection efficacy and microbiological safety. These methods mainly evaluate faecal contamination (e.g. *Escherichia coli* and Enterococci) thereby underestimating the actual number and diversity of microorganisms present. Recently, new fluorescence-based technologies offer significant advantages and overcome the limitation of conventional plate count methods. These alternative and more rapid diagnostic devices and techniques characterise a microbiological community and the efficacy of the disinfection process independent of cultivability within a matter of minutes to hours, thereby providing early warning of a contamination event. Furthermore, these alternative devices can be utilised on site by trained personnel thereby eliminating the requirement for the sample to be couriered to a specialised lab for analysis.

This report aims to assess these new devices and techniques to evaluate the effectiveness of disinfection technologies including chlorine based processes, peracetic acid, performic acid, H_2O_2 and UV and their applicability within water reuse schemes. The systems selected for evaluation of applicability include flow cytometry (FCM) and a molecular indicator system quantitative real time polymerase chain reaction (qPCR).

1.1 Objective

This deliverable aims to utilise these new devices and techniques to evaluate the effectiveness of disinfection technologies including chlorine based processes, peracetic acid, performic acid, H_2O_2 and UV and their applicability within water reuse schemes. The systems selected for evaluation of applicability include flow cytometry (FCM) and a molecular indicator system quantitative real time polymerase chain reaction (qPCR).

1.2 Selected devises and techniques

1.2.1 Flow cytometry

FCM offers real-time monitoring (within 15-20 minutes from sampling), ease of use (limited specialisation of the personnel), transportability, high precision, high reproducibility, low cost, and amenable of automation. The FCM represents a generic technology that counts and measures the optical properties of individual particles of 0.1 – 50 μm in a flow stream. The analysis is performed by measuring the scattered laser light (both forward (FSC) and side scatter (SSC)) and the fluorescence signals through photomultiplier detectors (PMTs) (Figure 1). Approximately 1,000 events per second can be detected within any sample that can be reduced to a suspension of mono-dispersed particles with a mass suitable for analysis.

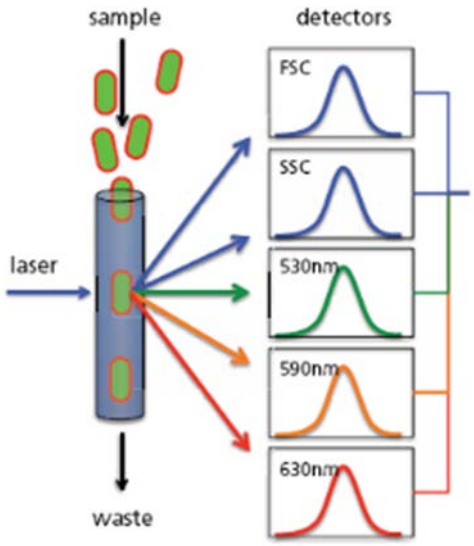


Figure 1 Schematic of the flow cytometry measuring and detection set-up

The method is based on staining microorganisms with fluorescent dyes which target a particular cell function and enable characterisation, e.g. cell viability, whilst differentiating cells from background particles in the same size range. Dyes typically intercalate into nucleic acids resulting in strong signals upon binding. Cells are subsequently focused in a stream of liquid (sheath fluid) and passed by an electronic detection system that records signals after exciting the corresponding dyes at suitable wavelengths. Fluorescent properties and scatter behaviour are recorded giving rise to 2-dimensional plots that allow the distinction of microbial sub-populations (every cell is visible as a dot in the plot).

1.2.2 qPCR

The real-time polymerase chain reaction is a laboratory technique based on the polymerase chain reaction (PCR). With the help of fluorescent dyes and a Real-Time Thermal Cycler it is possible to monitor the amplification of DNA molecules during the PCR (in real-time). With the application of specific predefined DNA-standards it is possible to quantify the DNA during this process. (Quantitative real-time PCR). There are several possible methods for real time PCR measurements, the one used in this project is based on non-specific fluorescent dyes that intercalate with any double-stranded DNA.

With the help of this method it is not only possible to reduce the time until results are available to a few hours, with the right equipment and trained personnel the tests can be performed directly on site. The more precise assessment of the microbiological risk of disinfected effluent waters, due to the possible inclusion of target organisms, which are non-culturable, is an additional advantage. In order to distinguish between live and dead cells a pre-treatment step with the DNA binding dye propidium monoazide (PMA) was performed on the samples. This effectively removes bacterial DNA of compromised cells, from subsequent DNA amplification and detection steps.

1.3 Demosites

The water recycling demosites selected for the FCM assessment represent a variety of multi-barrier processes, treating water sources of differing characteristics for the intended end point use of irrigation and non-potable activities including toilet flushing. These demosites are detailed further in sections 1.3.1 and 1.3.2. Additional waste water sites located in Berlin and Braunschweig, Germany and representing varying

disinfection procedures including performic acid, UV and/or ozone, were further selected for the assessment of the qPCR molecular biological tool.

1.3.1 Old Ford Water Recycling Plant, UK

1.3.1.1 Blackwater reuse treatment plant

The Old Ford Water Recycling Plant (OFWRP) began operation in 2012 to coincide with the London Olympic and Paralympic Games with the aim of contributing to the reduction of potable use for non-potable activities by 40% in alignment with the Olympic Delivery Authority's 'Sustainable Water Strategy'.

The OFWRP is the UK's largest community wastewater recycling scheme operated by Thames Water Utilities Limited (TWUL). A schematic of the OFWRP treatment process is provided in Figure 2. The OFWRP mines (abstracts) raw sewage, characterised as domestic and light commercial plus surface runoff, from the Northern Outfall Sewer designated for Beckton Sewage Treatment Works. The influent raw sewage is initially pre-treated within two underground septic tanks for the removal of rags and gross solids. The settled sewage is then pumped through two 1 mm rotating screen units for the removal of particulate matter, including hair and fibres, which could cause damage to the subsequent membrane process. Biological treatment is undertaken within a membrane bioreactor (MBR). The MBR comprises an activated sludge (AS) unit with a segregated anoxic and aerobic zone, followed by ultrafiltration (UF) membranes configured within 3 racks with a nominal pore size of 0.04 μm (Figure 2). Post-MBR treatment includes absorption through two granular activated carbon (GAC) filters operated in parallel for the removal of any remaining colour, prior to disinfection with sodium hypochlorite for a chlorine residual between 0.3 mg/L and 1.5 mg/L for storage within the reclaimed tank prior to distribution.

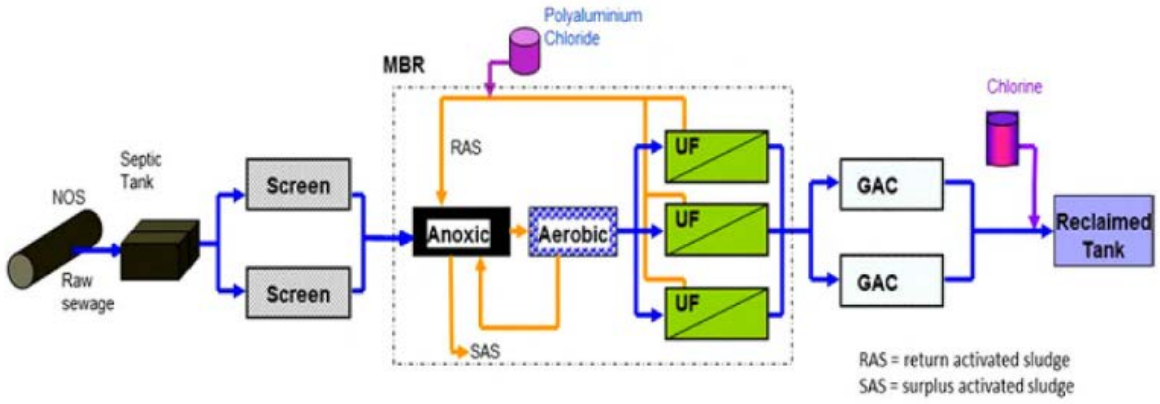


Figure 2 Schematic of the OFWRP treatment train (Hill and James, 2014)

1.3.1.2 Queen Elizabeth Olympic Park non-potable distribution network

The multi-barrier treatment process enables treatment to a unique non-potable standard for distribution throughout the Queen Elizabeth Olympic Park (QEOP) within a dedicated non-potable distribution network (thereby minimising the risk of cross connections) spanning 3.65 km and servings venues throughout the QEOP (Figure 3). End uses of the reclaimed product include WC flushing, irrigation and supplementing rainwater harvesting systems.

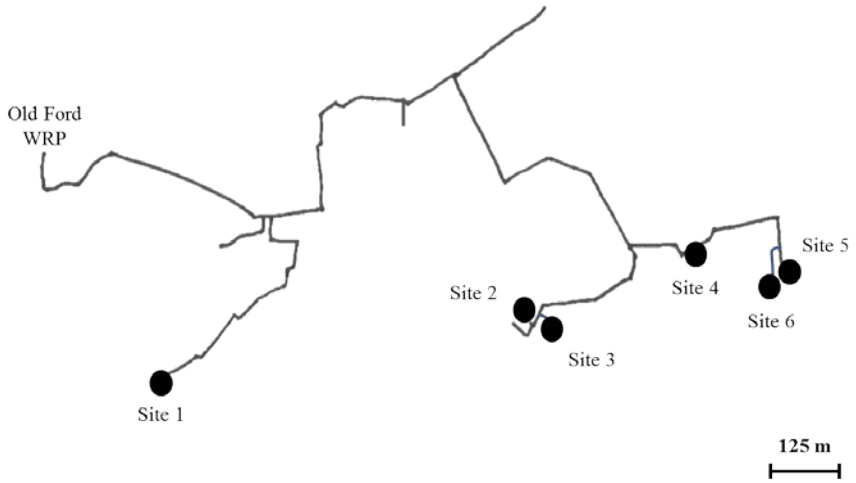


Figure 3 The QEOP non-potable distribution network and sampling points (not to scale).

The OFWRP is capable of an operational turnover of 574 m^3/d. Following the London 2012 Olympic Games (defined as the 'legacy' period) the OFWRP continues to operate and supplies the remaining customers within the QEOP, at a demand varying from approximately 600 m^3/d during the spring/summer period to 40 m^3/d during autumn/winter (Hill and James, 2014). Variation in demand observed with seasons is attributed to irrigation and increased visitor numbers to the QEOP during the spring/summer months, and reduced irrigation requirements and low visitor numbers in the autumn/winter. The reduction in demand observed during the autumn/winter has resulted in a lower network turnover and stagnation of the reclaimed water within the distribution network resulting in low Cl residuals.

1.3.2 "Capitanata", Italy

1.3.2.1 Wastewater treatment plant

"Capitanata" is located at Fiordelisi's premises and treats wastewater produced within the factory (on average about 140 m^3/d). The plant also treats wastewater collected from the factory's toilets (about 5-10 % of total wastewater), therefore the presence of faecal indicators in wastewater is relevant (on average 4.E+06 CFU/100mL of *E. coli*), even if lower (about one order of magnitude) than what is usually found in municipal wastewater.

The wastewater treatment plant, which is schematized in Figure 4, is based on an activated sludge process (pre-denitrification, nitrification, sedimentation). A fraction of the secondary effluent is further treated with pressure sand filtration (possibly preceded by a low chlorination), and membrane ultrafiltration (Kristal 600 ER – Hyflux - hollow fibre membranes, with 0.05 μm pore size), and sent to the test field for experimental crops irrigation. The percentage of secondary effluent that is treated by sand and membrane filtration can vary in the range 30-100 %, depending on the actual flow of the raw wastewater and on the performances of the filtration unit.

The operating flux obtained during the first months of operation was about 15 L/m^2/h, which is lower than the value provided by the manufacturer (50 L/m^2/h at 25°C for industrial wastewater). In order to match irrigation frequency with the continuous effluent production, a storage tank (10 m^3) is placed next to the test field, whose overflow is sent to a large storage basin and then to soil (where also the secondary effluent overflow goes). When irrigation is operated, water is withdrawn from the storage tank and sent to the field through an on-line UV disinfection system (6 mercury vapour lamps, 200W each) aimed at removing possible residual contamination or bacterial regrowth that may occur within the tank.

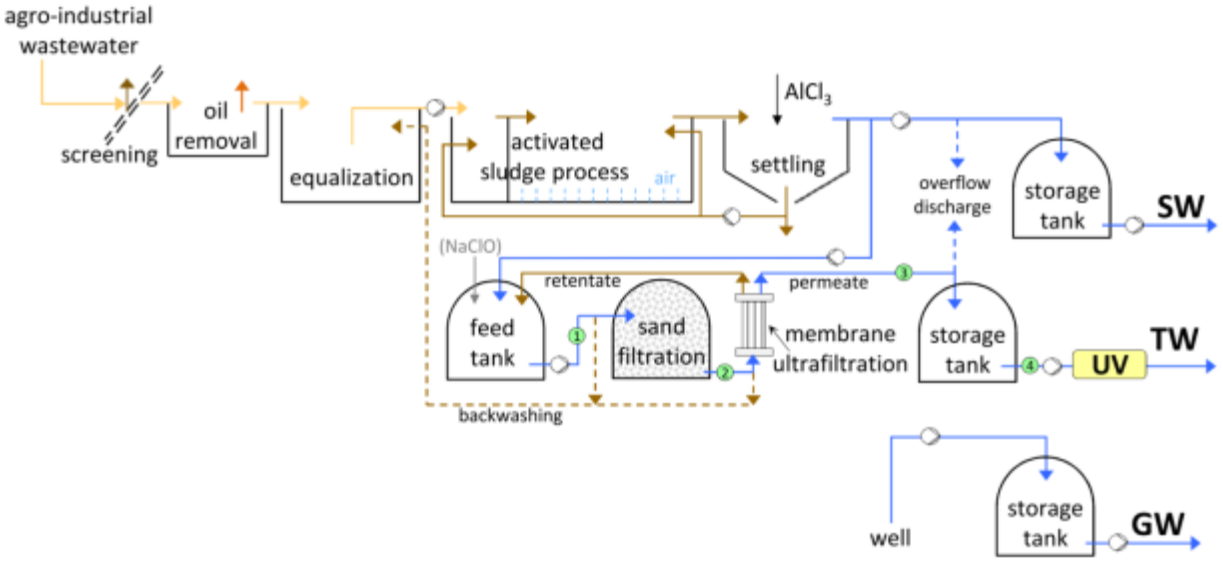


Figure 4 Configuration of the wastewater treatment plant operated at "Fiordelisi" company and sampling points.
The scheme shows the treatment processes that each type of irrigation water (SW, TW, and GW) underwent before being used in the field experiments, and the other samples points within the tertiary treatment plant.

1.3.2.2 Experimental irrigation plots

Three types of water are used for irrigation: secondary effluent (SW), tertiary effluent (TW), well water (GW) (see Figure 4). The experiment is laid in a randomized block design with each of type of irrigation water replicated three times. The crops are grown under a net house structure, covered with an anti-hail net, in nine identical 15 m x 30 m plots that are located near the wastewater treatment plant. The crops seedlings are transplanted into the plots in mulched paired rows spaced at 250 cm between each other, with the plants at a distance of 30 cm apart along each single row. Fertilization, pest and weed control are performed according to local management practices. The fruits are hand harvested at full stage maturity. A drip irrigation system, arranged in the middle of each paired row, is used for the crop irrigation. Irrigations are performed when the available soil moisture is depleted to the threshold value of 40%.

2 Materials and Methods

2.1 Microbiological characterisation of the Old Ford Water Reuse System

2.1.1 Flow cytometry

Data collected by UCRAN utilised an Accuri C6 flow cytometer (Becton Dickinson, UK), for the purpose of:

- Enumeration of total cells: SYBR Green I (SG I) penetrates both intact and damaged cells. Cells appear green upon appropriate excitation as a result. Green fluorescence is recorded in the FL1-channel.
- Enumeration of intact cells: in addition to SG I, propidium iodide (PI) is used as a second stain. PI does not penetrate intact cells, but only damaged ones. As a result cells stain red upon appropriate excitation. Red fluorescence is recorded in the FL-3 channel.
- Occurrence of the major microbial sub-populations (including HNA and LNA cells) (Figure 5)
- Live/Dead cells ratios before and after sanitation processes.

Applying the detailed staining procedures allows enumeration of total cell concentration (TCC) and intact cell concentration (ICC) and the relative proportion of intact cells. For chlorinated water distribution systems, the percentage of intact cells tends to correlate with the concentration of free chlorine in water (Gillespie et al. 2014). The 2D-plot generated shows red fluorescence (FL3) vs. green fluorescence (FL1) (Figure 5). Plotting these two parameters gives good resolution of different bacterial clusters based on their fluorescence staining behaviour.

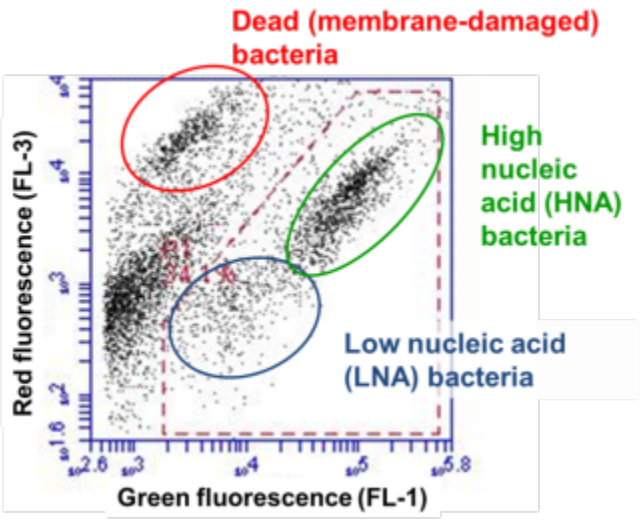


Figure 5 Example of a flow cytometric dot plot of a water sample stained with SGI and PI.
Intact bacteria are located in the gated region. Two distinct bacterial clusters can often be observed based on their nucleic acid content: high nucleic acid (HNA) and low nucleic acid (LNA) bacteria. Cells with damaged cell membranes are outside of the gated region. Other signals represent background of organic or inorganic origin.

2.1.2 Experimental plan

The OFWRP and non-potable distribution network were characterised through FCM, with the following research plans pursued:

- Monitoring the OFWRP treatment efficiency: samples taken at various points along the treatment train and analysed for TCC and ICC, with the effect of treatment assessed through cell enumeration and live:dead ratios.
- Microbiological map of distribution network: samples collected during the winter and summer (representing periods of low and high demand) to assess the change of microbial numbers with water age and regrowth potential. The TCC and ICC are further correlated with surrogate parameters within the non-potable distribution network.
- Effect of water stagnation: microbial concentrations compared in parts of the system with pronounced water consumption and stagnation, with the impacts of maintenance flushes on microbial concentrations and live:dead ratios assessed.

2.1.2.1 OFWRP treatment train sampling and analysis

Samples were collected every 1 -3 months via designated sampling taps throughout the treatment train enabling grab samples of raw sewage, screened sewage, MBR treated effluent (including anoxic, mixed liquor and post membrane), post-GAC, post-chlorine disinfection and final treated waters. Samples are collected within 100 mL sterile sample bottles (Aurora Scientific, UK). Bottle pre-dosed with thiosulfate are used for sample collection for processes following the disinfection stage to eliminate the chlorine residual. Samples were transported at 4^{o}C and analysed within 24 hours of collection.

Laboratory analysis at UCRAN included TCC and ICC through FCM following methodology as described by Gillespie et al. (2014) and outlined within section 2.1.1, and heterotrophic plate counts (HPCs) for the presence of coliforms and *E.coli* following dilution and filtration of a 1 mL sample through a 0.45 μm filter placed onto a MLGA plate (Membrane Lactose Glucuronide Agar), completed in triplicate, and incubated for 24 hours at 35^{o}C.

Online data provided by TWUL for the treatment train include pH, NTU, temperature and chlorine concentration, with in-house laboratory data provided for TOC and DOC.

2.1.2.2 Distribution network sampling and analysis

Samples from the distribution network were collected during periods characterising high and low demand (summer and winter) and following scheduled network flush events completed by TWUL as part of a network maintenance strategy. Six points throughout the 3.65 km network are sampled, varying in end point use and proximity to the OFWRP (Figure 3).

Grab samples for FCM analysis were collected in sterile 100 mL bottles pre-dosed with thiosulfate (Aurora Scientific, UK). Sampling was performed according to TWUL sampling guidelines, with samples collected prior to and post-tap flushing, both prior to and post a network maintenance flush event (Figure 6). Sampling post network flush occurred immediately after the maintenance flush in the winter, but within a week after the summer flush. Briefly, the tap was marginally opened and allowed to flush for 30 seconds and then sampled. The tap is then treated with concentrated sodium hypochlorite and fully opened to flush for a further 5 mins before collecting a post-flush sample. Grab samples for assimilable organic carbon (AOC) analysis measured through regrowth potential are also collected prior to and post-tap flush within AOC free 30 mL glass vials pre-dosed with thiosulfate. All samples are transported to the laboratory at 4^{o}C

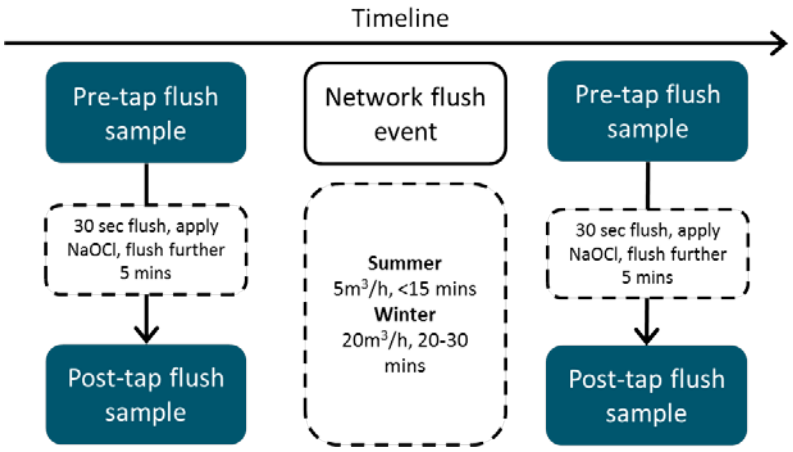


Figure 6 Schematic representation of sampling scenarios for individual sample taps.

Laboratory analysis at UCRAN included FCM analysis of TCC and ICC within 24 hours of collection (as previously described); and AOC through regrowth monitored via FCM following incubation for 7 days at 22°C as detailed within Gillespie et al. (2014).

Free and total chlorine was analysed at the time of sampling using a Compact Chlorometer Duo (Palintest, UK), following the manufacturers guidelines and temperature monitored using a Traceable digital thermometer (Fisher Scientific, UK). In-house laboratory data provided by TWUL for sampling events include DOC, TOC, total nitrogen (TN), phosphorus (P), suspended solids (SS) and turbidity; in addition to standard microbial analysis through HPCs for coliforms and *E.coli*. Water age/residence time is estimated through TWUL online metering data (reported in m3/d) was received from the automated meter readings (AMR) and acquired through the online database FUSION for all sampled points within the network.

2.1.3 Statistical analysis

The Principal Component Analysis (PCA) was used to reduce the multiparametric cytometric dataset to only two variables accounting for as much as possible of the total variance. The biplots, computed by the data correlation matrix, allowed to graphically summarizing the patterns of cytometric variables at each treatment step. All variables were normalized using division by their standard deviations.

2.2 Verifying the fate of microbial contaminants in agricultural irrigation schemes

2.2.1 Flow cytometry

For microbiological analyses of the sampled waters, IRSA-CNR used a compact flow cytometer (Apogee A50-Micro) suitable to be transported on-site (< 30 kg). This newly-available machine was specifically developed for microbiological analysis, preserving a precise optical alignment after transportation.

In this task, we mainly concentrated on the following:

- Enumeration of total aquatic microbes (including prokaryotes, protists and microbial aggregates)
- Occurrence of the major microbial sub-populations (including HNA, LNA cells)
- Single cell size and biomass (according to the mean light scatter signals)
- Mean fluorescence signals as a proxy of cell activity
- Live/Dead cells ratio before and after sanitation processes.

The aquatic microbes are identified using a single cell cytometric approach to highlight their different morphological, structural and functional properties (Boi et al., 2016). An example of a typical flow cytometric protocol is shown in Figure 7.

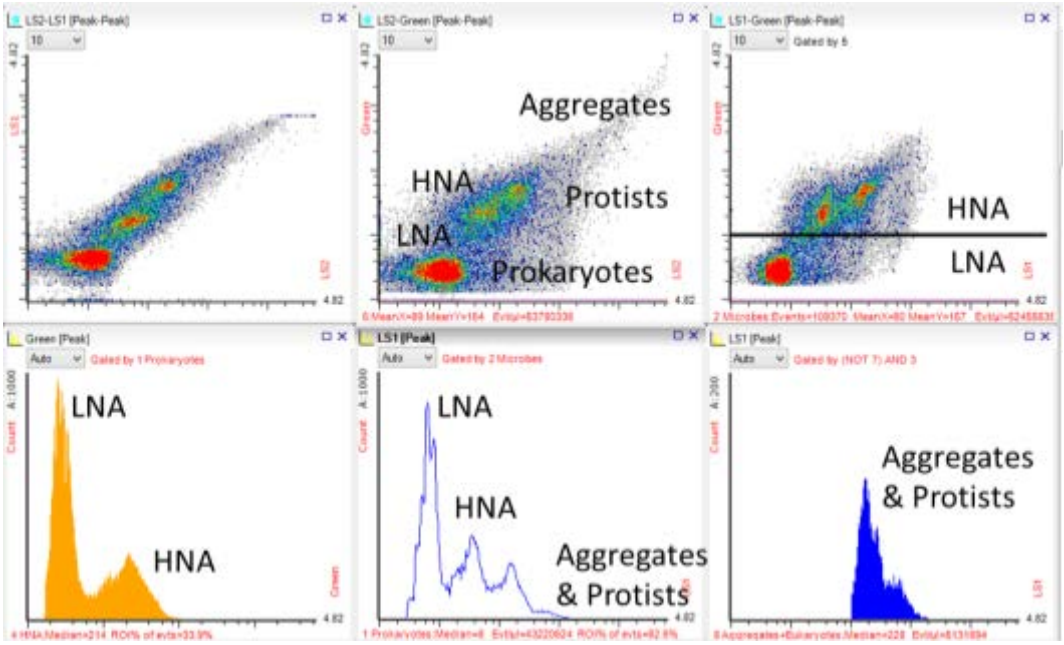


Figure 7 Example of a dot plot panel of a water sample stained with SYBR Green I.
Different microbial sub-population can be observed based on their nucleic acid content (green fluorescence signal) and size (forward and side light scatter; LS1, LS2), including low nucleic acid (LNA) and high nucleic acid (HNA) prokaryotic cells, microbial aggregates and protists

2.2.2 Faecal indicators

Microbiological methods adopted at the "Capitanata" demosite include the determination of *Escherichia coli* and *Salmonella* spp., serving as indicators of faecal contamination, as required by Italian national regulations for effluents reuse in irrigation. Faecal Coliforms were also measured in order to evaluate the possible contamination of soil and plants.

For the enumeration of *E. coli* in wastewater samples, the Colilert® method (IDEXX Laboratories) was used (ISO 9308-2:2012). It is based on the β-D-glucuronidase activity, which is known to be characteristic of *E. coli* and few other bacterial species. Therefore, Colilert does not need the confirmation of presumptive *E. coli*. This makes both the execution and the interpretation of Colilert analysis faster and easier than common cultivation-based methods (membrane filtration and pour plate). Moreover, Colilert, as other enzymatic methods, detect viable (and not only cultivable) bacteria, so avoiding the possible significant underestimation that may occur in cultivation-based methods. The IDEXX Colilert® method uses the patented Defined Substrate Technology® (DST®). This makes use of the enzymatic reactions of particular groups or species of microbes. Two enzyme substrates are included in the Colilert test: the chromogen o-nitrophenyl-β-D-galapyranoside (ONPG) which is converted by the enzyme (β-galactosidase) found in coliforms and whose reaction releases o-nitrophenol responsible for the yellow colour; the fluorogen 4-methyl-umbelliferyl glucoronide (MUG) that is cleaved by an enzyme found in *E. coli* (β-glucuronidase). After 18-24 h incubation at 35 ± 0.5°C, a total-coliform-positive reaction turns the medium yellow. An *E. coli*-positive reaction causes the medium to fluoresce under a long-wave ultraviolet light (366 nm). It is rapid (18-24h).

Salmonella spp. can normally survive in the environment. The presence of *Salmonella* in domestic wastewater varies in concentration depending on population pathologies. For these reasons its occurrence in water basins is considered a biological indicator of a clear dangerous faecal contamination. For *Salmonella* spp. determinations a Biosafety Level 2 laboratory is needed. Analyses of *Salmonella* spp are performed by subcontractor Università di Foggia following the standard ISO 19250:2013. This method requires some consecutive steps:

1) Pre-enrichment (to recover damaged cells);
2) Enrichment (to increase bacterial load of Salmonella);
3) Isolation (only Salmonella's colonies survive);
4) Eventual biochemical and serological confirmations.

Analyses of *E. coli* on matrices different from water (soil and crops) are performed within the same contract. These analyses are conducted by the spread plate method, as follows: 25 g of each sample is added to 225 mL buffered peptone water, homogenized in a stomacher for 180 s, and stored at room temperature for 30 min to allow bacterial cell recovery. Then serial 10-fold dilutions in buffered peptone water are spread onto plates containing TBX agar. The plates are incubated at 37°C for 24 h.

Faecal Coliforms are measured following the spread plate method, but using a C-EC agar.

2.2.3 "Capitanata" sampling and sample preparation

A monthly sampling campaign was performed between May 2014 and May 2016. This included the treated effluents used for irrigation, other wastewater samples within the tertiary treatment process (points 1 to 4 in Figure 4), samples of soil irrigated with the different types of irrigation water. Crops (fruits) are taken only during harvesting period. Grab sampling is always performed. The analyses of faecal indicators (*E. coli* and *Salmonella* spp) are performed within 4 hours from sampling. For FCM, fixation is executed by adding formaldehyde (2 % final concentration). However, FCM is also executed (on site) on unfixed samples every second month.

An automated monitoring system acquires data related to the tertiary treatment performances. These are: inlet pressure to sand filtration; outlet pressure of sand filtration (which is also the inlet of membrane filtration); flow; suspended solids concentration at the influent of sand filtration. Data are recorded every half an hour.

2.2.4 Statistical analysis

Mann-Whitney U test was applied to verify the statistical significance of the difference between two sets of consecutive treatments (i.e., In vs SF; SF vs UF; UF vs Tank; Tank vs UV).

The PCA was used to reduce the multiparametric cytometric dataset to only two variables accounting for as much as possible of the total variance. The biplots, computed by the data correlation matrix, allowed to graphically summarizing the patterns of cytometric variables at each treatment step. All variables were normalized using division by their standard deviations.

2.3 Molecular biological tools for the detection, identification and quantification of microbial contamination

2.3.1 Sampling of different waste water treatment plants

2.3.1.1 Water samples (preparation and transport)

To investigate the influence of different disinfection steps, we collected water samples in waste water treatment plants in Berlin and Braunschweig. These sites performed disinfection procedures with performic acid, UV and/or ozone. The samples were collected before and after the different disinfection steps, either with subsequent transport (cooled) into the laboratory or filtration directly on site and subsequently transported (cooled) to the laboratory. In Berlin, the waste water treatment plant Ruhleben disinfected with 18 ppm performic acid, 9 min; and UV treatment: 110 Mw/s * cm^2. In Münchehofe there was a disinfection with ozone: 0.94 mg O_3 / mg DOC, 18.5 min. The waste water treatment plant in Braunschweig disinfected with performic acid (2 ppm, 20 min) as well as with UV treatment (35 Wh/m^3, 7.3 s).

2.3.1.2 Biofilm samples (preparation and transport)

Besides water samples, also biofilm samplings were performed. Therefore, we exposed a perforated sampling device, filled with glass beads, at which the biofilm-forming bacteria adhere (Figure 8). The biofilm devices were exposed in the waste water treatment plant Münchehofe in Berlin for two weeks. To investigate the influence of disinfection on biofilm-forming bacteria, the biofilm devices were transported (cooled) to the laboratory and disinfected with 2 ppm peracetic acid for 10 min.

Figure 8 Biofilm device, glass beads are inside the white body

2.3.2 Molecular methods

2.3.2.1 DNA extraction

DNA extraction was completed within 1- 4 hours of sampling using the EURx GeneMATRIX Soil DNA Purification Kit was used. The Kit comprises of a mechanical and chemical cell lysis step. Followed by a washing step and the DNA-binding step.

By vortexing the glass beads, the attached biofilm was removed in a physiological buffer. 500 µl of the bacteria suspension were used for the following DNA extraction step. For the DNA-Extraction of filtrated samples the filter was put in bead tubes, which were included in the EURx GeneMATRIX Soil DNA Purification Kit. The DNA was eluted in elution buffer and stored at – 20 °C.

2.3.2.2 PCR

Collected DNA was amplified with polymerase chain reaction (PCR). For amplification the 16s rDNA for sequencing, universal bacterial primer 63f and 1387r were used, for deep-sequencing, the modified primerset 341f and 541r, respectively (Marchesi et al, 1998; Klindworth et al, 2012). The EURx Taq PCR Master Mix, and a PCR Cycler (Bio-Rad) was used for the amplification of the DNA-Samples. Afterwards the amplified PCR-product was checked by gel electrophoresis.

2.3.2.3 qPCR

Quantitative real-time PCR can be used to quantify the number of specific DNA-copies in an unknown DNA-sample. By applying a fluorescent stain, a subsequent gel electrophoresis is no longer necessary, the amplification of a target DNA molecule can be monitored in real time. With the help of standard DNA the initial concentration of the target DNA can be calculated. In this project, the fluorescence stain SYBR Green, present in the EURx SG qPCR Master Mix (Roboklon), and the CFX96 Touch Real Time PCR Detection System (BioRad) were used. In this method a DNA-binding dye binds to all double-stranded (ds) DNA in a PCR, causing fluorescence of the dye, which can be detected by a combination of LEDs and detectors within the qPCR device. An increase in DNA product during the PCR leads to an increase in fluorescence intensity measured in comparison to a DNA standard at each cycle. Because the dye will bind only to double stranded DNA, it is of special importance to design a robust PCR essay and prevent the formation of primer dimers, which could produce false signals.

2.3.2.4 Gel-electrophoresis

By gel electrophoresis molecules like DNA, RNA and proteins can be separated according to their size and charge. In molecular biology this method is used to analyze a mixed population of DNA / RNA fragments by length to estimate their size. By applying an electric field, DNA and RNA fragments move through a matrix of agarose with small and big pores, driven by their negative charge. Corresponding to their length, small DNA or RNA fragments will move faster through the agarose matrix than a bigger fragment. Thus, the method serves as a possibility to separate nucleic acid fragments by length and therefore to analyze PCR products after amplification of DNA (Carle and Olson, 1984).

2.3.2.5 Sequencing and cloning

After amplification of the DNA of those bacteria that remained viable after different applications of chemical disinfection (see Section 2.3.4.2) a population analysis was performed by cloning and sequencing. This served as an isolation step for the DNA of viable bacteria and allowed for a determination of those indicator bacteria that are the most resistant to the specific treatment process. In addition the isolated DNA fragments generated by the cloning procedure were used for the development of DNA standards for the treatment efficiency indicator system. To retrieve even more in depth sequence data from a mixture of DNA in a sample, 454 sequencing was applied. In this process the DNA is first amplified with universal bacteria primers which contain specific oligonucleotide adaptors. Each DNA fragment is attached to a bead and the beads are PCR amplified within droplets of an oil–water emulsion. This generates multiple copies of the same DNA sequence on each bead. After this, the beads are captured in picolitre-sized wells in a fabricated substrate and pyrosequencing (pyrophosphate-based sequencing) is performed in parallel on each DNA fragment as shown in Figure 9.

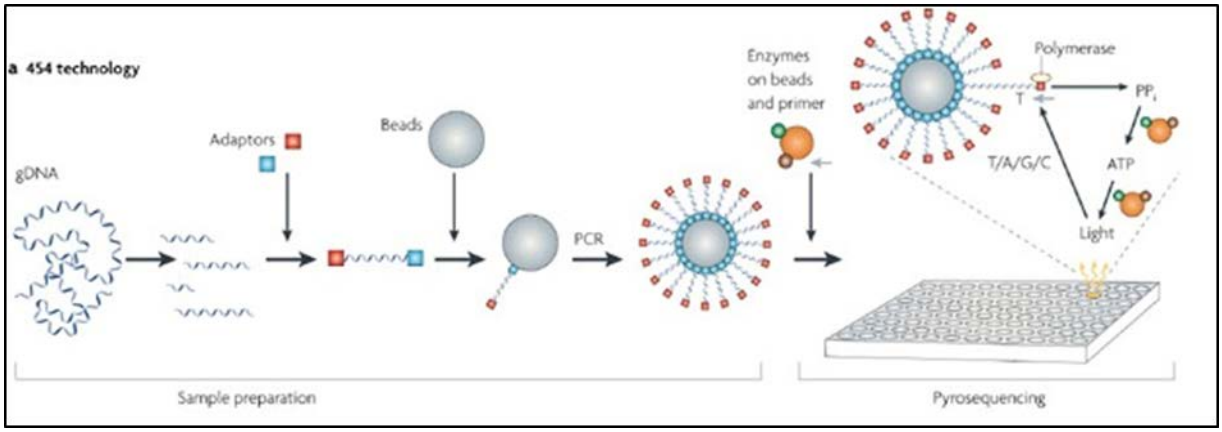


Figure 9 454 sequencing procedure (Medini et al, 2008)

Nucleotide incorporation is detected by the release of inorganic pyrophosphate (PPi), which leads to the enzymatic generation of photons: PPi is released and converted to ATP and luciferase uses the ATP to generate light. The cycle is iteratively repeated for each of the four bases. The average read length is more than 600 bp (Medini et al 2008). This method helps to gain a very detailed look into the community profile of PMA treated samples. By comparing the results of treated and untreated samples, relevant indicator organisms can be revealed.

2.3.3 Determination of indicator organisms

2.3.3.1 Different disinfection in waste water treatment plants

Collected samples (after different disinfection processes) were analyzed by two approaches (Figure 10). Approach A starts with a microbiological step: cultivation by selective mediums and the isolation of viable organisms. For isolation, the selective media Brilliance *E.coli* / coliform selective agar, Legionella GVPC selective medium GVPC, Pseudomonas Cetrimide (Thermo Scientific) agar as well as non-selective media like plate count agar and Tryptone soya broth (Thermo Scientific) were used. Afterwards the DNA-extraction (Section 2.3.2.1) and the amplification via PCR followed. The sequencing process was the terminal step in Approach A. There was a downside to this approach, because it isn´t possible to identify non-cultivable organisms this way. There is a high probability of non-cultivable organisms, which are more resilient to pass disinfection steps than certain cultivable organisms. Therefore it would have been possible to miss important indicators. Approach B prevented these disadvantages by applying PMA to identify only intact cells. By using cloning and sequencing in approach B, dominant organisms in the population could be determined. In addition DNA fragments which were useful for the creation of standards for the indicator system emerged.

<table>
<tr><td></td><td colspan="2">Approach A</td><td colspan="2">Approach B</td></tr>
<tr><td>Disinfection</td><td colspan="4">as applied in full-scale or pilot plant or dedicated lab-protocol</td></tr>
<tr><td>Identify survivors</td><td colspan="2">Cultivation and Isolation</td><td colspan="2">Filtration and PMA treatment</td></tr>
<tr><td rowspan="3"></td><td colspan="4">DNA extraction</td></tr>
<tr><td>DNA amplification with universal primerset</td><td>DNA amplification with special Deep Sequencing pri-merset</td><td>DNA amplification with universal primerset</td><td>DNA amplification with special Deep Sequencing pri-merset</td></tr>
<tr><td>Cloning or Se-quencing</td><td>Deep Sequencing</td><td>Cloning or Se-quencing</td><td>Deep Sequencing</td></tr>
<tr><td>Quantify survivors</td><td colspan="4">qPCR methods
(primer development, standard DNA)</td></tr>
</table>

Figure 10 Methods used to identify indicator organisms

2.3.3.2 Analysis of sequencing data

The software MEGA 6 and the online database NCBI were used to analyse the received sequence data. To identify regions of similarity in the sequence data, they were included into a sequence alignment. The similarities in specific DNA regions are a consequence of an evolutionary relationship. To find out, which bacteria are in the investigated population, the sequence data was first aligned and sequences with high evolutionary similarity were compared with known sequence data in the online database NCBI.

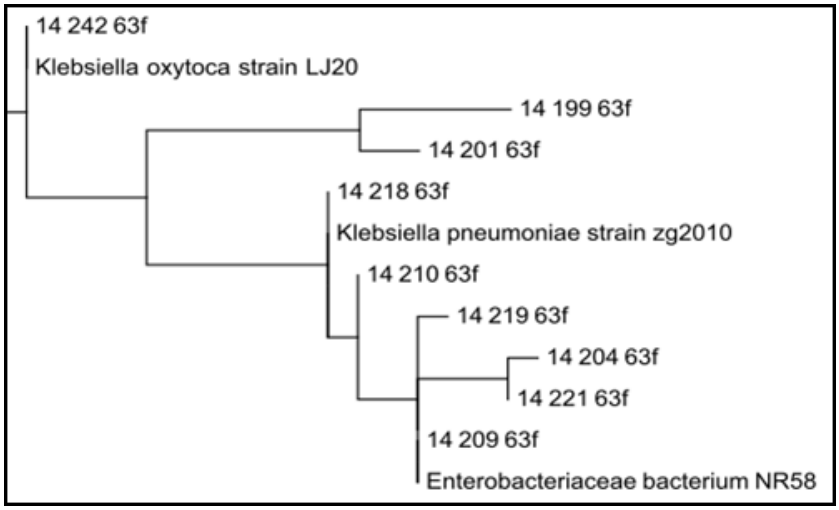


Figure 11 Example of a section of a phylogenetic tree of DNA sequences, samples collected following UV treatment

Figure 11 represents a subsection of a phylogeny tree, based on analysis of the 16s region, encoding a vital part of prokaryotic ribosomes. Due to its slow evolution rate, the prokaryotic 16s region is very conservative and is used in molecular phylogeny. To identify the organisms in the sample, DNA sequences were compared to the existing sequences in the online databases.

2.3.4 Development of indicator system

2.3.4.1 Primer design and standard DNA

With the help of the reference DNA collected in the cloning and sequencing step, a qPCR assay was created for the quantitative detection of the chosen (resistant) indicator bacteria. Therefore, a standard DNA for each indicator organism, as well as a specific primer set was needed. The reference DNA, which was collected in the cloning and sequencing steps, served as a starting point for the production of standard DNA. Using universal primer sets, standard DNA fragments were amplified by PCR, cleaned up by gel electrophoresis and stored at -20 °C for the further development of the indicator system. For the development of specific primer sets a literature research gave the first impulses. By creating alignments for each indicator organism, primer design could be verified and optimized. Also, online database like NCBI and SILVA were used to check the specificity of each developed primer. The further qPCR-product, which also is regulated by the selected primer, is an important point. All chosen primers bind at the 16s region of bacteria and were tested for performance. The development could allow for a very fast process control of the disinfection step in a treatment plant. With the help of a multi-step protocol, DNA of viable indicator bacteria can be quantified and help in the assessment of the success and safety of the treatment of the effluent water.

2.3.4.2 Live-dead investigation by propidium monoazide

When applying molecular methods like polymerase chain reaction (PCR) to environmental samples, one of the most prevalent problems is that, in contrast to microbiological methods, a distinction between viable (live) and nonviable (dead) cells cannot be accomplished easily. Dead and living cells both contain DNA, which can be detected with molecular methods. Propidium monoazide (PMA) is a photoreactive DNA-binding dye that can be used to inactivate the DNA of compromised (nonviable) bacterial cells prior to PCR. The dye passes only the cell walls of those bacteria that have been damaged by the disinfectant and a specific LED light can be used to induce a photoreaction between the PMA and the DNA that will

lead to a covalent bond (Figure 12). This will render the DNA non-amplifiable by PCR. Living organisms do not react with PMA, as they have an intact cell membrane. After treatment with PMA only the DNA of living bacteria remains amplifiable by PCR, bound PMA inhibits the subsequent amplification step (Bae and Wuertz, 2009; Nocker *et al*, 2007). Thus, the PMA treatment provides a differentiation between live and dead cells. Unfortunately, there is a supposed disadvantage of the PMA-treatment: UV-treated cells can probably not be analysed, because of the non-compromised membrane. For that reason, dead cells with DNA-damage by UV-treatment could still be amplifiable by PCR. However, a successful differentiation between live and dead cells by PMA and a subsequent qPCR is published (Lee, E. et al, 2015). Thus, UV treated cells were stained with PMA and the amplificability was checked via qPCR (Figure 12).

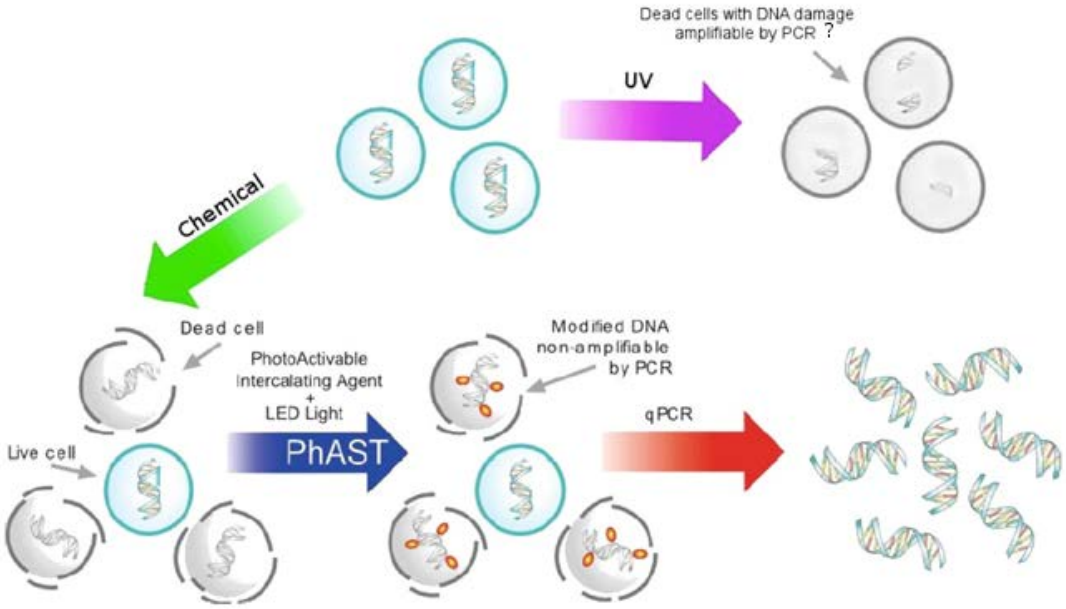


Figure 12 PMA treatment procedure and the covalent binding at the DNA of chemically disinfected cells (Source: modified after www.genuil.com).

2.3.5 Adaptation of procedure to pilot plantPilot project – testing of indicator detection system

2.3.5.1 Optimisation of PMA treatment protocol

The PMA treatment depends on two parameters – the used concentration of PMA and the exposure time. PMA concentrations ranging from 2µM to 100 µM in combination with different exposure times (5 to 40 minutes) were tested. The transparency of the samples is also important for the photo activation step: Samples with high turbidity may not perform successfully, if they are not diluted enough. For further analysis, a **PMA concentration of 50 µM and an exposure time of 15 min were determined**. Turbid samples should be diluted before the photo activation step.

2.3.5.2 Transport of samples and construction on an in-field blue LED incubator

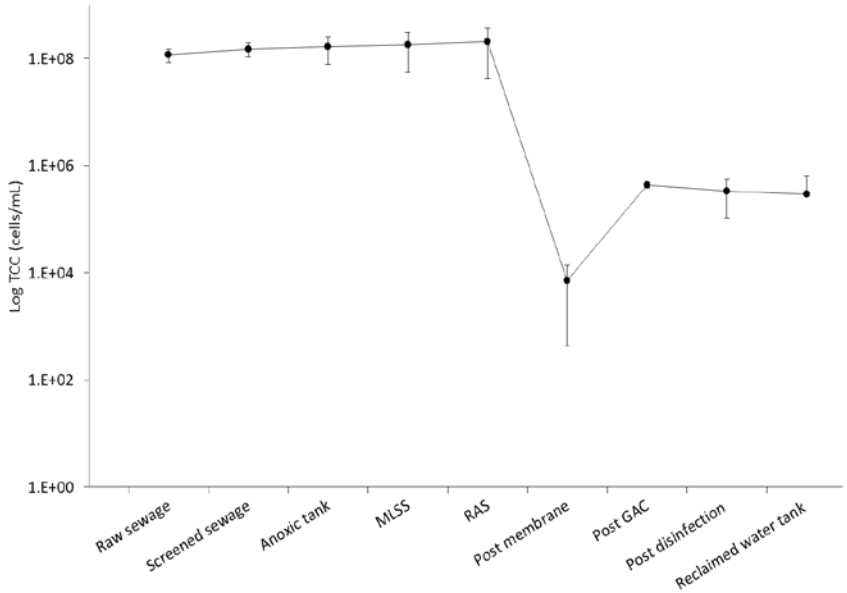
At first sampling, samples taken before and after different disinfection steps were transported (cooled) to the laboratory and subsequently filtered and treated with PMA. Also a one-site filtration was realized. However, to minimize an influence on the viability of cells, a PMA treatment should be applied as soon as possible. Therefore, a transportable LED incubator was constructed. As a consequence samples could be treated with PMA directly after collection and an additional filtration step is not required.

3 Results

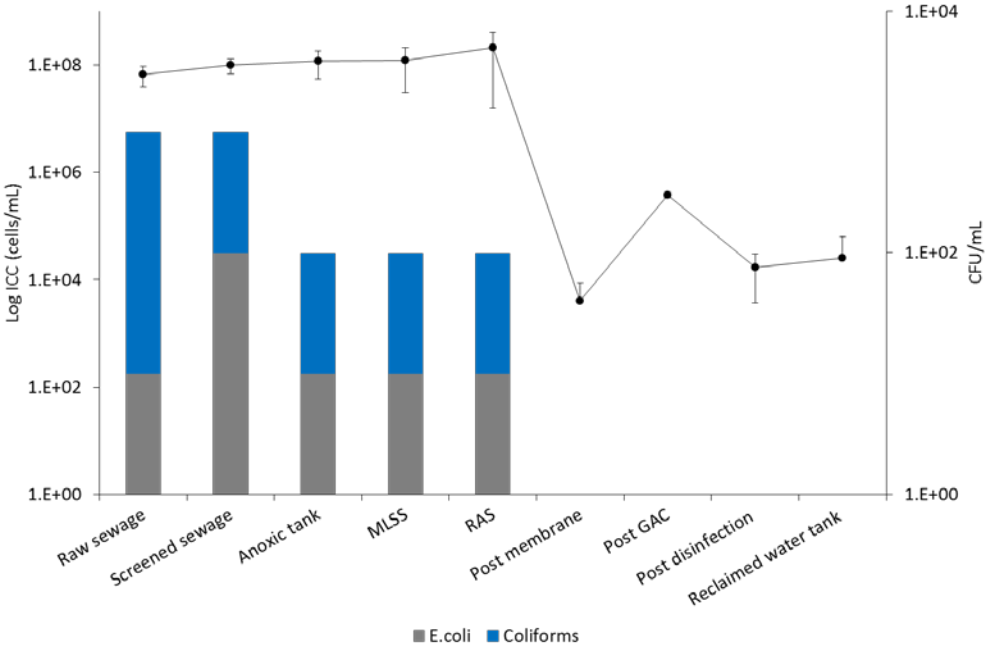
3.1 Microbiological characterisation of the Old Ford Water Reuse System

3.1.1 OFWRP treatment process characterisation

Analysis of the TCC for the treatment train throughout the sampling campaign resulted in an initial cell concentration of approximately 10^8 cells/mL within the influent raw sewage (Figure 13a).



a) TCC



b) ICC

Figure 13 Average cell concentration for the OFWRP treatment train for a) TCC and b) ICC including HPC for presumptive coliforms including *E.coli* **(mean ± standard deviation) (Whitton et al. 2016).**

In contrast to expectations, the TCC remained consistent throughout the treatment train until post-MBR. Extending the gate within the FCM software to capture counts characterised by a higher fluorescence within the FL-1 and FL-3 channels, did not significantly change the results (data not shown). This is likely due to aggregation of particles through the creation of flocs within the MLSS, RAS etc. which may require further sample preparation prior to analysis (i.e. vortex/sonication). A 4 log unit reduction was observed following ultrafiltration to a TCC of approximately $7x10^3$ cells/mL (Figure 13a). The TCC was then found to increase following the GAC through a re-release of biological material from the filters to an approximate concentration of $4x10^5$ cells/mL. The re-release of biological material following GAC treatment is expected as the filters represent a biological treatment process. Following the GAC, the TCC remained consistent for the remaining treatment process finalised by storage within a reclaimed water tank prior to distribution (Figure 13a).

The accompanying ICC for samples taken throughout the treatment train during the project were found to follow the TCC with no significant deviations in measured concentrations until post-disinfection by chlorination (Figure 13b). Following disinfection the ICC decreased as expected, representing approximately 4.4% and 9.3% of the TCC sampled post-disinfection and within the reclaimed water tank respectively (Figure 13b). Overall the final reclaimed water was characterised by a 3 log unit reduction in ICC in comparison to the influent raw sewage, with results consistent throughout the project irrespective of when sampling occurred i.e. summer or winter.

Examination of the FCM 2D plots enables characterisation of the bacterial community throughout the OFWRP treatment process through the observable bacterial clusters for the determination of communities characterised as HNA and LNA (Figure 14). Those bacterial populations characterised by a HNA profile represent the actively growing proportion of the population, suggesting the environment and conditions are favourable to growth, productivity and associated treatment.

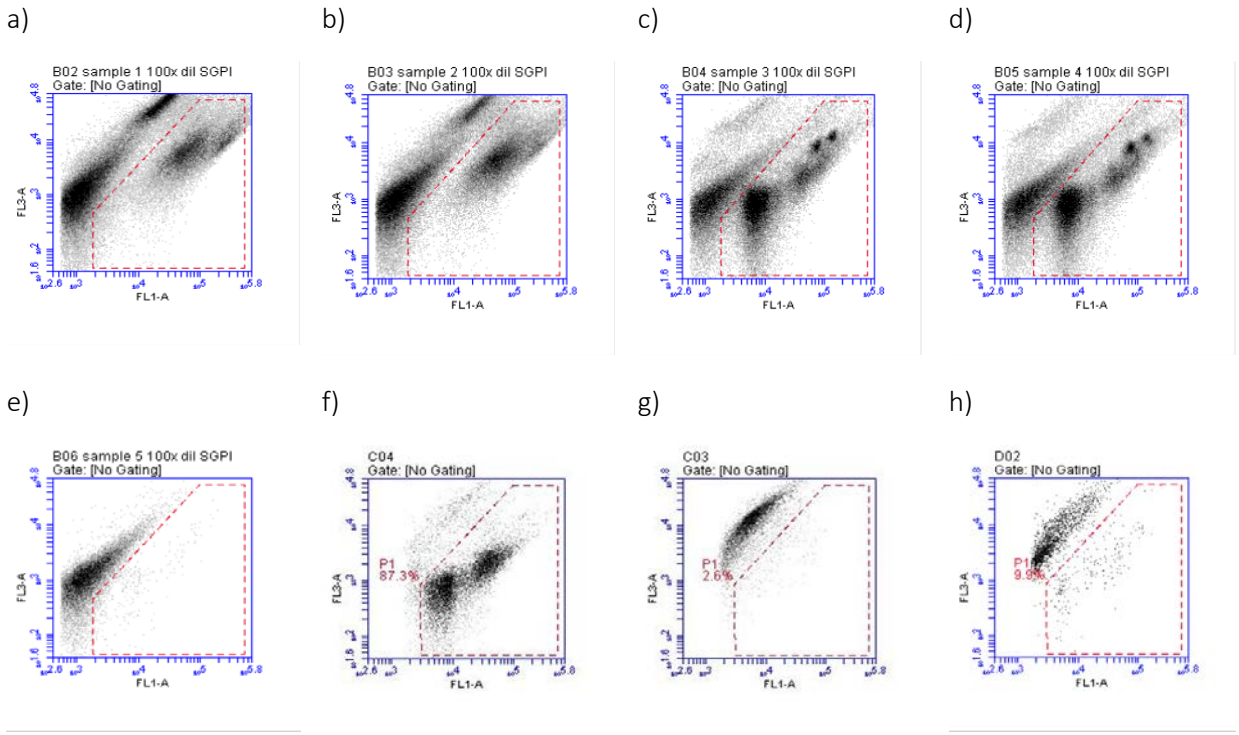


Figure 14 ICC FCM fingerprints for the OFWRP treatment process for a) influent raw sewage, b) screened sewage, c) anoxic zone of MBR, d) MLSS, e) post-MBR, f) post-GAC, g) post-disinfection and h) reclaimed storage tank (Whitton et al. 2016).

The 2D plots initially find the bacterial community within the raw and screened sewage to be characterised by predominantly HNA bacterial community representing 83.6% and 81.9% of the intact population respectively (Figure 14a and b). However, a shift in this profile to a predominately LNA characterised community representing 66.3% and 67.3% of the microbiological community is observed from the anoxic zone of the MBR and within the MLSS (Figure 14c and d).

The shift in bacterial profile is understandable upon reflection of the design and operation of the biological treatment process of the MBR. The design of this system was based on a higher strength sewage typically encountered during the summer period. The reduction in sewage strength received by the OFWRP during the autumn/winter has resulted in an extremely long sludge age (approx. 1,000 days) which is reflected by the increase in LNA population. Whilst the OFWRP is meeting treatment standards throughout the year, TWUL are currently investing in a programme of research to further enhance performance as current operational conditions are unlikely to be optimal as reflected by a LNA bacterial community through FCM. Following biological treatment, the intact bacterial community is representative of a balanced LNA and HNA population (Figure 14f) prior to disinfection and the reduction in an ICC prior to distribution.

These observations in community profile were further confirmed through PCA analysis of the TCC and ICC, with cytometric similarities observed between; 1) influent raw sewage and screened sewage, 2) anoxic, MLSS and returned activated sludge (RAS) and 3) post-disinfection and the reclaimed storage tank (Figure 15).

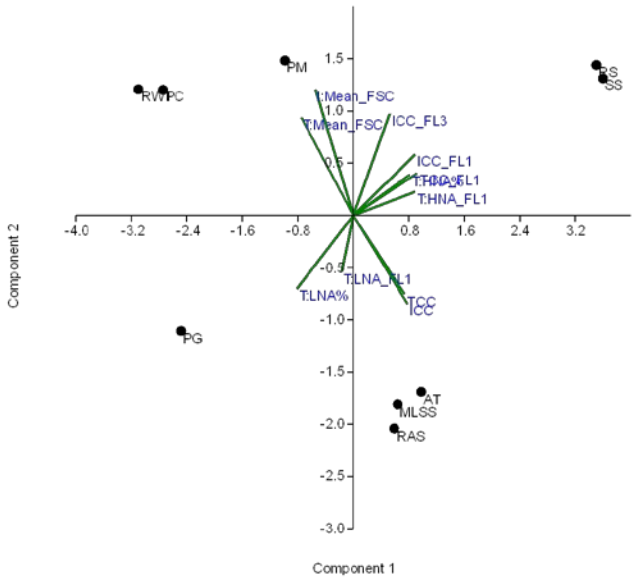


Figure 15 Biplots of the OFWRP PCA for median values of TCC and ICC carried out via the data correlation matrix.
(RS) Raw sewage, (SS) settled sewage, (AT) anoxic tank, (MLSS) mixed liquid suspended solids, (RAS) returned activated sludge, (PM) post-membrane, (PG) post-GAC, (PC) post chlorination and (RW) reclaimed water.

The results observed through FCM for bacterial enumeration were also confirmed through laboratory HPC analysis. HPC data confirmed the presence of coliforms including *E.coli* within the raw sewage, screened sewage and the biological component of the MBR process prior to ultrafiltration. Plate counts observed a decrease in CFU/mL from approximately 10^3 to 10^2 from the screened sewage to MBR biologi-

cal treatment (Figure 13b) with no viable counts detected following ultrafiltration, thereby further confirming the efficacy of the ultrafiltration process and the bacterial reduction observed through FCM analysis.

Overall, the findings through FCM analysis are believed representative of the microbiological community throughout the OFWRP treatment process. The consistency of results demonstrated throughout the course of the research provides a baseline reading for the treatment process, with deviations and increases in counts indicative of problems within the process which may require addressing. As such, FCM can be utilised by operators to monitor the performance of assets within the treatment train, with long term assessment identifying deterioration in performance with increasing asset life and maintenance requirements, and unexpected peaks/spikes in cell numbers (e.g. greater than one standard deviation) indicative of immediate issues and the requirement of corrective actions.

3.1.2 Queen Elizabeth Olympic Park Non-potable distribution network

3.1.2.1 Non-potable network characterisation, including stagnation and network flushing activities

Sampling and characterisation of the QEOP non-potable distribution network was completed during period's representative of high consumer demand (summer) and low consumer demand (winter). Samples were taken for a combination of routine maintenance activities including prior to and post individual taps flushes, and prior to and post network maintenance flushes as described in section 2.1.2.2.

Analysis of the ICC prior to a network flush and tap flush were found to increase with distance from the OFWRP, with the most distal points within each branch of the network characterised by a ICC >4 x10^5 cells/mL for both the summer and winter sampling campaign (i.e. Zone 2, Zone 6, Timber Lodge, Zone 7 and Eton Manor) (Table 1 and Table 2) and representative of approximately a 93.5% increase in ICC when compared to the reclaimed product distributed from the OFWRP. Following a tap flush, the ICC was found to decrease in the majority of cases by between 4.0% and 96.9% during the summer and 32.4% and 81.3% in the winter. However, in the case of Zone 7 and Eton Manor the ICC was found to increase following a tap flush in the winter period by 7.4% and 17.5% respectively (Table 2). As such, a tap flush was found to unsuitable for these points during winter through the increased ICC observed through stagnation at these points.

Table 1 ICC for the QEOP non-potable distribution network with increasing distance from the OFWRP sampled following tap and network maintenance flushes during the summer period representing a high demand.

Sample tap location	Pre network flush, pre tap flush	Pre-network flush, post-tap flush	Post-network flush, pre-tap flush	Post-network flush, post-tap flush
Site 1	4.61 x 10^5	1.42 x 10^4	3.80 x 10^5	8.50 x 10^3
Site 2	3.18 x 10^5	1.18 x 10^4	1.13 x 10^5	1.51 x 10^5
Site 3	4.75 x 10^5	1.49 x 10^4	2.15 x 10^5	2.91 x 10^4
Site 4	2.46 x 10^5	2.19 x 10^4	6.54 x 10^4	1.98 x 10^4
Site 5	5.01 x 10^5	4.81 x 10^5	2.07 x 10^5	2.57 x 10^5
Site 6	6.50 x 10^5	5.19 x 10^5	1.04 x 10^6	4.01 x 10^5

Table 2 ICC for the QEOP non-potable distribution network with increasing distance from the OFWRP sampled following tap and network maintenance flushes during the winter period representing a low demand.

Sample tap location	Pre network flush, pre tap flush	Pre-network flush, post-tap flush	Post-network flush, pre-tap flush	Post-network flush, post-tap flush
Site 1	3.80×10^4	1.70×10^4	1.30×10^4	NS
Site 2	7.83×10^5	5.29×10^5	3.00×10^4	NS
Site 3	2.84×10^5	1.83×10^5	2.30×10^4	NS
Site 4	2.30×10^5	4.30×10^4	3.30×10^4	NS
Site 5	4.03×10^5	4.35×10^5	2.80×10^4	NS
Site 6	5.61×10^5	6.80×10^5	3.30×10^4	NS

NS = Not sampled

Comparisons of the ICC of samples taken prior to a tap flush, for pre and post-network flush, demonstrate a noticeable decrease, with reductions in the range of 17.4% to 73.4% in summer and 65.8% to 96.2% in the winter (Table 1and Table 2). The greater reduction in ICC during the winter flush is associated to the enhanced network flush procedure undertaken during this period (Figure 6), characterised by an increased flushing velocity associated to enhanced physical removal and biofilm scouring.

Analysis of the proportion (%) of intact cells in comparison to the total concentration further confirms the significance of network flushing activities with reductions ranging 2% to 32% in the summer, and 7% and 38% in the winter (Table 3). The points exhibiting the lowest reductions for both seasons are Site 5 and Site 6 . These sample points are located at the furthest point on the network from the OFWRP. The network flush schedule is typically completed on the branches of the network in the order of Site 1, Site 3 and Site 7. . As such, an increase in cell concentration is believed to be a consequence of upstream flushing and the accumulation of biomass at the distal point of the network. These results suggest a greater flush velocity and/or volume should be adopted to ensure reductions in intact proportions aligned to the rest of the network, with the adoption of bespoke flushing procedures for network points possible through flow cytometric analysis.

Table 3 Percentage ICC prior to and following network maintenance flushes completed in the summer and winter.

Sample tap location	Summer		Winter	
	%ICC prior to network flush	%ICC post-network flush	%ICC prior to network flus	%ICC post network flush
Site 1	45	13	71	33
Site 2	42	29	79	57
Site 3	49	18	61	43
Site 4	40	21	71	41
Site 5	47	45	83	77
Site 6	49	47	80	73

Despite a proportion of intact cells of up to 49% in the summer, and 83% in the winter prior to the network maintenance flush, HPC data confirms the absence of presumptive coliforms including *E.coli* (data not shown) The intact cell population is therefore believed to consist predominantly of heterotrophic

bacteria posing no risk to public health. The impact of this bacterial community on water aesthetics is further analysed in section 3.1.2.2.3.

3.1.2.2 Water quality parameters and ICC

Analysis was further undertaken to determine whether the water quality parameters of the reclaimed product within the distribution network could be correlated to the ICC and used as surrogate parameters for microbiological water quality. Furthermore, knowledge of the factors which support an increase in intact or 'alive' cells within the distribution network provides useful information regarding remediation strategies such as enhanced treatment at the OFWRP and/or network maintenance activities.

3.1.2.2.1 Dissolved organic carbon, total nitrogen, soluble reactive phosphorus and total suspended solids

Organic carbon is typically identified as the growth-limiting nutrient for heterotrophic bacteria and as such considered an important water quality parameter for the control of bacterial growth within a distribution network (Prest et al. 2016). Analysis for all samples taken from the QEOP non-potable distribution network throughout the project maintained a DOC concentration between 2.5 mg/L and 3.8 mg/L. The associated ICC of these samples were not found to increase with increasing DOC concentration within this range, with a decrease in ICC in comparison to the ICC within the reclaimed product of 30% at a DOC of 2.6 mg/L, in comparison to a maximum increase of 3,600% at a DOC of 3.1 mg/L (Figure 16a).

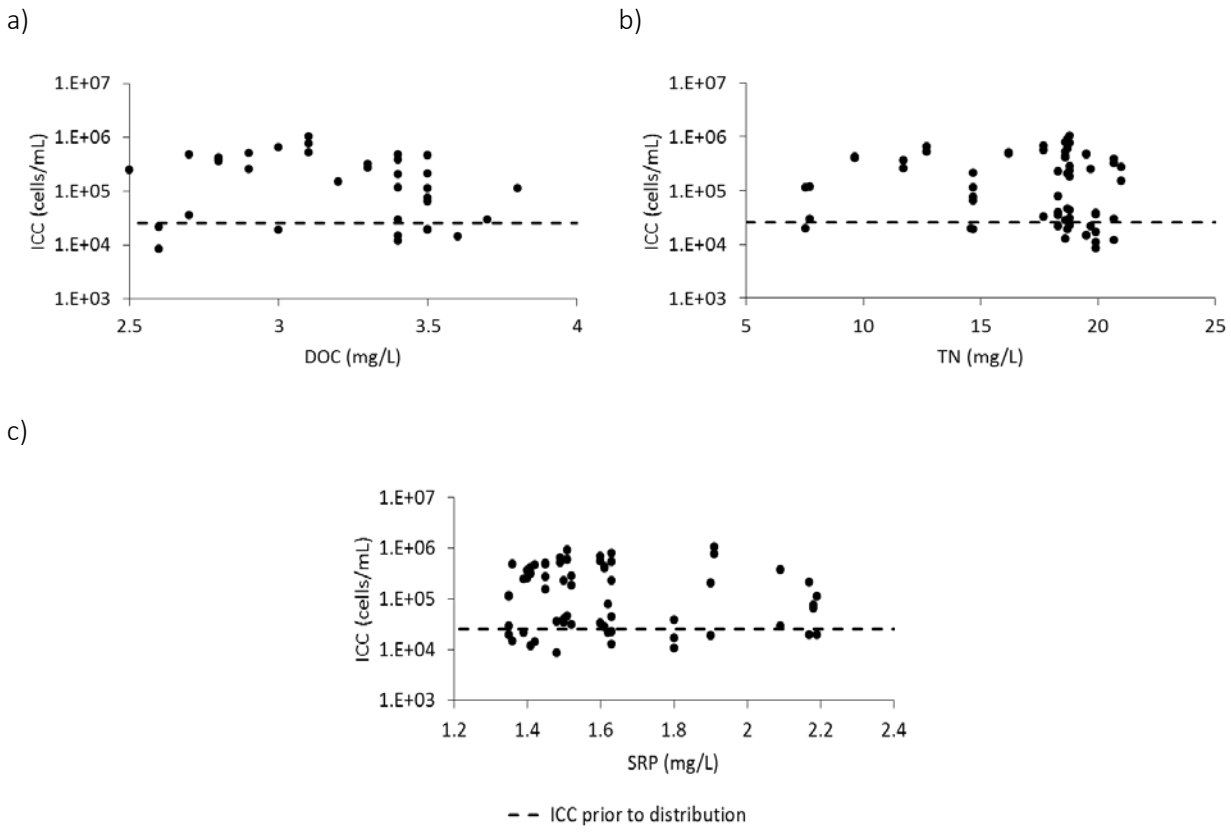


Figure 16 ICC of the non-potable distribution network samples and the corresponding water characteristics for a) DOC, b) TN and c) SRP (Whitton et al. 2016).

In addition to carbon (DOC); nitrogen (N) and phosphorus (P) are further described as essential nutrients for bacterial growth. Water quality analysis was performed to determine the total nitrogen (TN) and soluble reactive phosphorus (SRP) concentrations of the reclaimed water within the distribution network.

Similar observations were found to DOC, with no observable increase in ICC within increasing nutrient concentration for TN ranging from 7.5 mg/L to 21 mg/L (Figure 16b) and SRP ranging from 1.4 mg/L to 2.2 mg/L (Figure 16c). Analysis of the molar C:N:P ratios, commonly analysed for optima bacterial growth, similarly showed no relationship to ICC (data not shown). As such, the nutrient water quality of the reclaimed water is not believed to be the predominate parameters contributing to the observed increased of ICC within the distribution network and cannot be utilised by operators as a surrogate measurement to estimate microbiological water quality as determined through FCM for the QEOP non-potable distribution network.

Furthermore, the total suspended solids (TSS) concentration within the distribution network consistently remained below 2 mg/L, with no correlation observed in cell concentration with TSS (data not shown).

3.1.2.2.2 Free chlorine residual

In the case of chlorinated distribution networks, the percentage of ICC tends to correlate with the concentration of free chlorine (Cl) (Gillespie et al. 2014). Analysis of ICC concentration of the QEOP non-potable distribution network and free Cl for samples taken throughout the project, further confirm the findings of Gillespie et al. (2014). Results find the ICC to be greater than the average ICC within the reclaimed storage tank prior to distribution (approx. 3 x 10^4 cells/mL) at free Cl concentrations approximately < 0.34 mg/L (Figure 17). An increase in the Cl residual > 0.34 mg/L was observed to further reduce the ICC to 1.1 x10^4 cells/mL, representing a concentration 68% below the average ICC prior to distribution.

These findings therefore recommend a free Cl residual of > 0.34 mg/L should be maintained within the non-potable distribution network to limit the increase in viable bacteria within the reclaimed water with the free Cl concentration a suitable surrogate parameter which can be measured by operators to determine microbiological water quality within the distribution network. However, the target free Cl residual prior to distribution from the OFWRP is set at 0.6 mg/L. Typically, the further reduction of a free Cl residual within a distribution network is influenced through a combination of factors including water age and temperature.

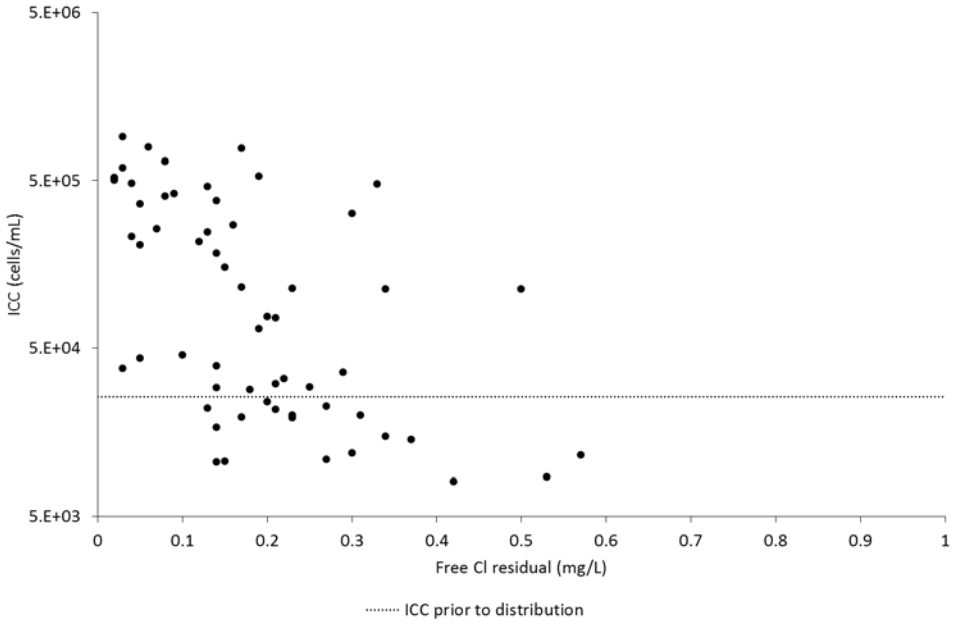


Figure 17 ICC within the non-potable distribution network in relation to the free Cl residual (Whitton et al. 2016).

A comparison of the free Cl residual and approximate water age of the samples within the non-potable distribution network (estimated through pipe volume and metred daily average water use), confirms a decreasing Cl residual with water age (Figure 17). When fixing the y intercept at the mean target free Cl residual for the OFWRP of 0.6 mg/L (target > 0.3 mg/L, < 1.5 mg/L (Hill and James, 2014), a decrease in residual to < 0.34 mg/L is predicted to occur at a water age of approximately 14 days. As such, results suggest in periods of low demand and stagnation, a network flush is recommended every 14 days (fortnightly) to prevent a build of microbiological biomass and deterioration of water quality. The flushing period estimated through this analysis remains consistent with TWUL current recommended flushing frequency.

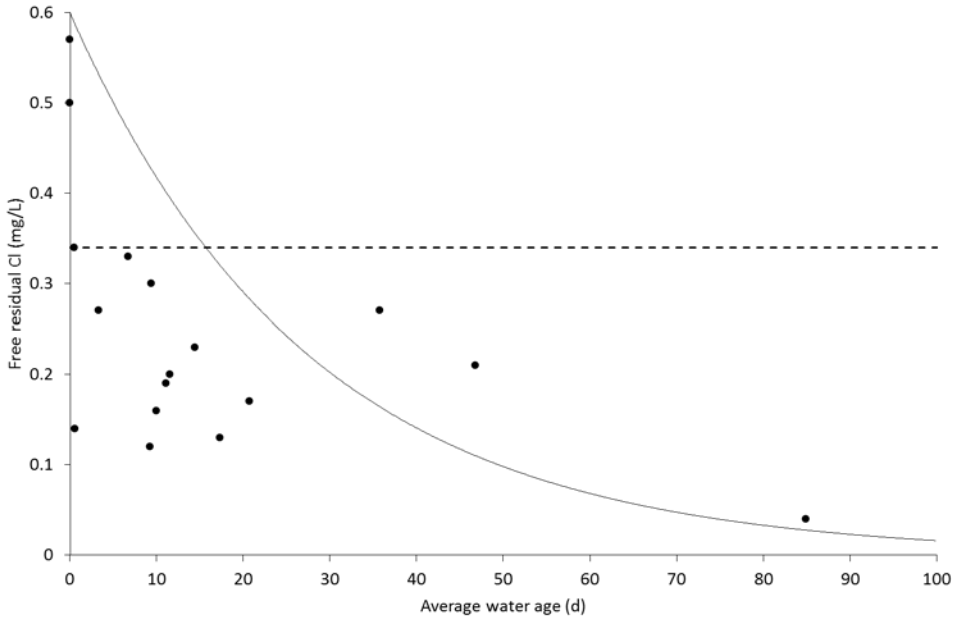


Figure 18 Reduction in residual Cl residual with average water age calculated for samples throughout the non-potable distribution network (y intercept fixed at 0.6 mg/L as the OFWRP target free Cl residual prior to distribution) (Whitton et al. 2016).

Unlike water age, no observable relationship was observed between the free Cl residual and the temperature of the sample upon collection for a range of temperatures between 6.9°C and 26.9°C (data not shown). The reduction in Cl residual cannot therefore be predicted in relation to season, and the associated increases and decreases in water temperature within the distribution network. As such, water temperature is not a suitable surrogate parameter for the determination of microbiological water quality.

3.1.2.2.3 Aesthetic impacts

Whilst an increase in bacterial numbers was observed within the non-potable distribution network, as a consequence of water age and Cl residual, standard microbiology analysis confirmed the absence of coliforms including *E.coli* thereby posing no significant risk to public health. An increase and proliferation of bacteria within the reclaimed product can however result in unfavourable aesthetic characteristics viewed negatively by the consumer. These qualities include turbidity, colour and smell which were assessed as surrogate parameters for water quality.

Laboratory analysis of samples collected from the non-potable distribution network, both prior to and post network flushes, confirmed the yellow hue (when compared to the platinum/cobalt scale) to be within the predetermined water quality standard of < 20mg/L (Knight et al. 2012). Colour ranged be-

tween 1.1 mg/L Pt/Co to 4.4 mg/L Pt/Co throughout the project over a range of TCC of approximately 2.8 x10^4 cells/mL to 1.2 x 10^6 cells/mL (Figure 19a). Results did not find an increase in the yellow hue quality of the reclaimed product with increasing cell concentrations.

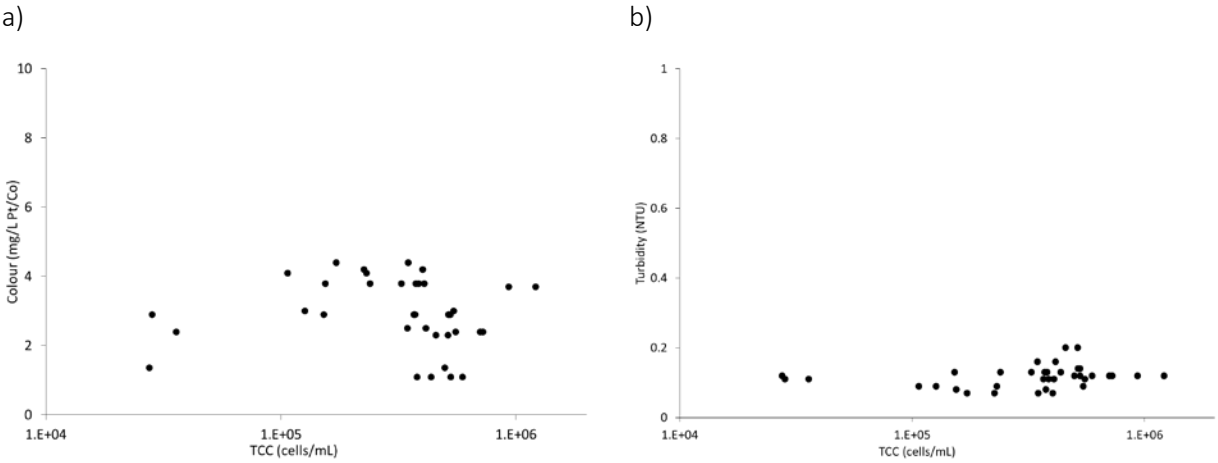


Figure 19 Water quality parameters effecting aesthetics and relationship to TCC for a) turbidity and b) colour (Whitton et al. 2016).

Similar observations were also found for turbidity, with all samples analysed below the water quality standard of < 2 NTU (Knight et al. 2012), with turbidity values ranging from < 0.06 NTU to 0.2 NTU, with no relationship observed between turbidity and TCC.

Furthermore, the possibility of unpleasant odour was analysed trough the concentration of dissolved sulphide. Increased levels of hydrogen sulphide (up to 1 mg/L) within a water distribution system are contributed to the decomposition of bacterial biomass and/or the bacterial reduction of sulphate under anaerobic conditions, resulting in an unpleasant odour. All samples collected from the non-potable distribution network during the project confirmed a sulphide concentration below the detection limits of the analytical methodology of < 10 µg/L, well below the OFWRP prescribed water quality standard of 50 µg/L (Knight et al. 2012) (data not shown). Overall results confirm the bacterial community present within the non-potable distribution network exhibit no unpleasant aesthetic qualities over the period of sampling regardless of the nature of the network flush nor frequency.

3.1.2.3 Regrowth potential within the non-potable distribution network

The implications of negligible Cl residual and stagnation were further investigated through a regrowth analysis, where the residual Cl was removed from the distribution network samples prior to incubating the samples at room temperature for a period of 7 days to observe the change in ICC through regrowth of the community utilising nutrients within the reclaimed product in addition to those released by the compromised biomass (Figure 20).

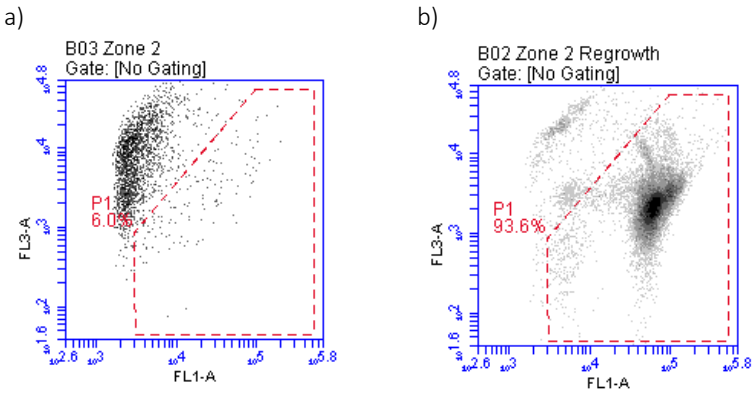


Figure 20 Example of re-growth observed for the network sample with the lowest initial ICC prior to incubation for regrowth, a) prior to regrowth and b) re-analysis after a period of 7 days and the conversion of compromised biomass into intact biomass (Whitton et al. 2016).

Results were found to be variable and demonstrated a maximum regrowth increase of up to 1.3 x10^7 cells/mL (~460 fold increase) with those samples with the lowest initial ICC demonstrating the greatest increase in ICC during the regrowth period (Figure 21). As such, network points with a negligible residual Cl have the potential to increase the intact biomass concentration by approximately 3,800% over a 7 day period through stagnation and negligible network activity or maintenance flushing.

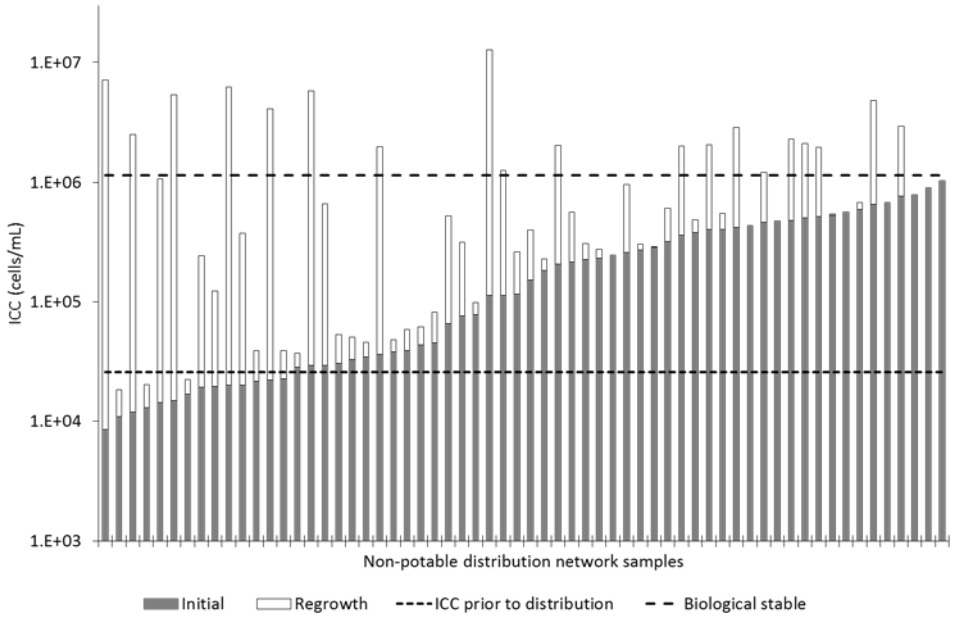


Figure 21 Regrowth potential of community within non-potable distribution network following 7 days, presented by increasing initial ICC (Whitton et al. 2016).

Characterisation of the microbiological water quality of the reclaimed product through FCM within the QEOP non-potable distribution network enables bespoke recommendations on network operation and maintenance; in addition to the identification of surrogate parameters which can be monitored by operators to ensure optimal water quality.

3.2 Verifying the fate of microbial contaminants in agricultural irrigation schemes

3.2.1 Flow cytometry

The results of the first monitoring campaigns, performed at the beginning of 2014, indicated the presence of suspended solids and faecal contamination (up to 1000 MPN/100mL of Escherichia coli) in the membranes permeate, suggesting a possible leakage. Membrane integrity was then evaluated by a pressure decay test (ASTM 2003), which indicated that 6 modules out of 8 presented relevant leakages. The first FCM monitoring survey was conducted on 11/04/2014. The corresponding results, obtained on-site within 2h from sampling, indicated different performances for microbial biomass filtration by the eight ultrafiltration modules. The prokaryotic abundance in the inflow water was 7.3 ± 0.4 x 10^6 cells/mL, while it ranged between 0.5 and 4.6 x 10^5cells/mL, respectively measured in waters outflowing from the UF1 and UF7 (Figure 22).

The higher log removal values (LRV) of aquatic prokaryotes were measured at UF1 (LRV = 2.2) and at UF2 (LRV = 1.9), confirming PDT results. The modules showing lower LRV, probably due to a different damage level, were isolated and the operating conditions of the entire system were remodulated to operate only with the two best-performing ultrafiltration modules (i.e., UF1 and UF2).

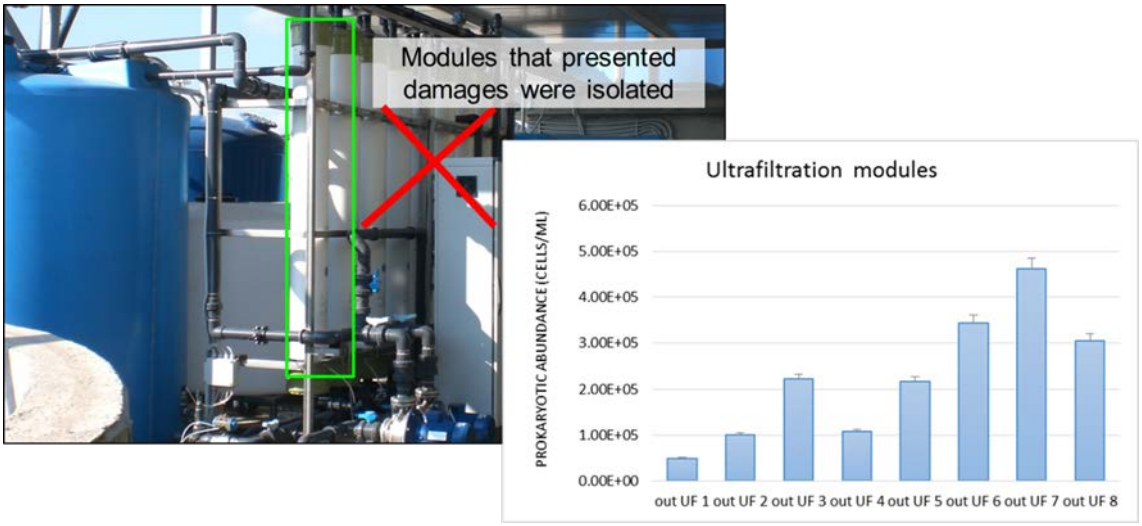


Figure 22 Prokaryotic cell abundance, measured by FCM at the outflow of the eight ultrafiltration modules (UF1-8). Error bars indicate the mean deviation of duplicates. Given the same inflow water inputs, different log removal values of aquatic prokaryotes could be related to different damage level of the modules.

The tertiary treatment plant operated with such downgraded configuration from May 2014 to September 2014, when all modules were then replaced with new ones. The flow cytometric monitoring along the treatment train continued until May 2016. Therefore, we could compare the efficiencies of applied treatments between the lowered and full operating conditions by assessing relevant cytometric parameters.

The aquatic prokaryotes represented the most abundant component in all water samples, with values ranging up to two orders of magnitude and with relevant differences among treatments. The protozoa, specifically identified as heterotrophic nanoflagellates, were approximately within a ratio of 1:1000 with the prokaryotic cells they feed on. Occasionally, their density was below the detection limit of the instruments (i.e., 10^2 cells/mL). The presence of particulate debris in suspension was also quantifiable by FCM. Overall, the different treatments affected the abundance of cells and particles circulating along the

treatment train (Figure 23). The dotted lines refer to the median values measured in well water, which is the conventional source of water used for crop irrigation.

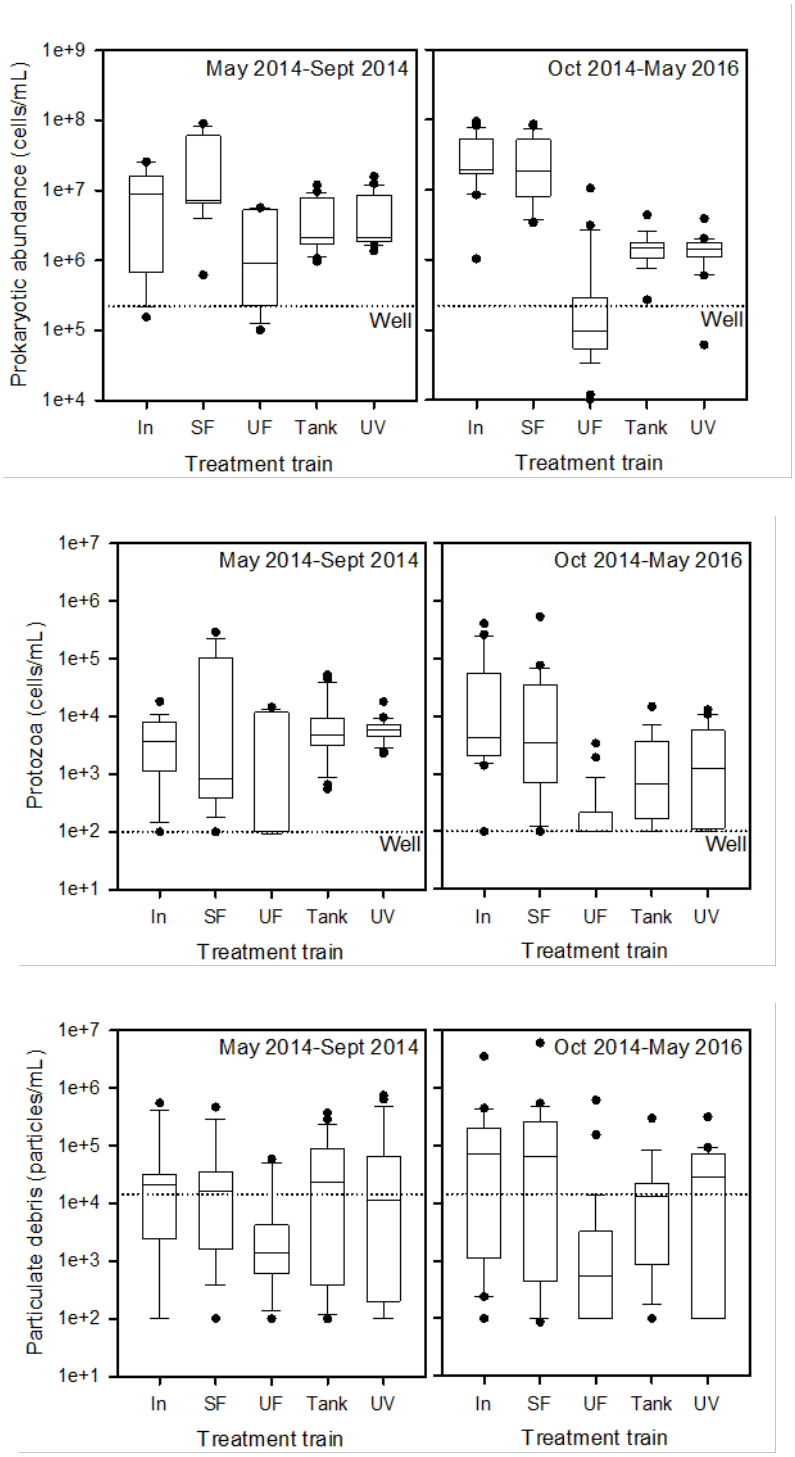


Figure 23 Box-plots of the abundance values of prokaryotes, protozoa and particulate debris, assessed by FCM before and after replacement of the ultrafiltration modules.

Lines within the boxes, boundaries, whiskers and dots mark the median, 25th &75th percentiles, 10th &90th percentiles, and outliers, respectively. The Cleveland linear interpolation method was used to compute the percentile values. The dotted lines refer to the median value in well waters alternatively used for crop irrigation.

In both periods, the sand filtration unit and, particularly, the ultrafiltration modules reduced significantly the abundance of prokaryotes and protozoa in the outflow waters. However, a significantly higher prokaryotic abundance was observed in waters collected from the following tanks, thus suggesting a consistent cell regrowth during storage period. A less marked regrowth pattern was observed for protozoa. The UV irradiation treatment did not produce any significant change in cell abundance. The particulate debris showed a significant decrease only when the new modules were operating (Table 4). Despite the above described variations were verified for both sampling periods, cell removal improved significantly when ultrafiltration was performed with the new intact modules (i.e., Oct 2014-May 2016).

Table 4 Statistical comparison of samples collected along the treatment train (see Figure 23).

The p-values indicate the statistical significance of the difference in the median values between the two adjacent treatments. p<0.001 = ***; 0.001<p<0.01 = **; 0.01<p<0.05 = *; p>0.05 = no statistical difference.

Mann-Whitney U Statistic (p-value)		
Prokaryotic Abundance	**May 2014-Sept 2014**	**Oct 2014-May 2016**
In vs SF	0.534	0.347
SF vs UF	<0.001***	<0.001***
UF vs Tank	0.01*	<0.001***
Tank vs UV	0.616	0.584
Protozoa Abundance	**May 2014-Sept 2014**	**Oct 2014-May 2016**
In vs SF	0.694	0.269
SF vs UF	0.027*	<0.001***
UF vs Tank	0.008**	0.003**
Tank vs UV	0.820	0.843
Debris	**May 2014-Sept 2014**	**Oct 2014-May 2016**
In vs SF	0.901	0.817
SF vs UF	0.085	0.014**
UF vs Tank	0.196	0.008**
Tank vs UV	0.563	0.378

During lowered operating conditions (May 2014-Sept 014), the two active ultrafiltration modules could remove on average 87% and 88% of prokaryotic cells and protozoa, respectively. Owing to the observed cell regrowth in the storage tanks, the overall prokaryotes removal by the tertiary treatment plant decreased to 75%. During full operating conditions (Oct 2014-May 2016), the UF cell removal was 99% for prokaryotes and 92% for protozoa, with slightly lower percentages when considering the entire system

(i.e., overall prokaryotes removal = 89%). The calculations of LRV offered a synthetic view of the system performances within the sampling periods (Figure 24).

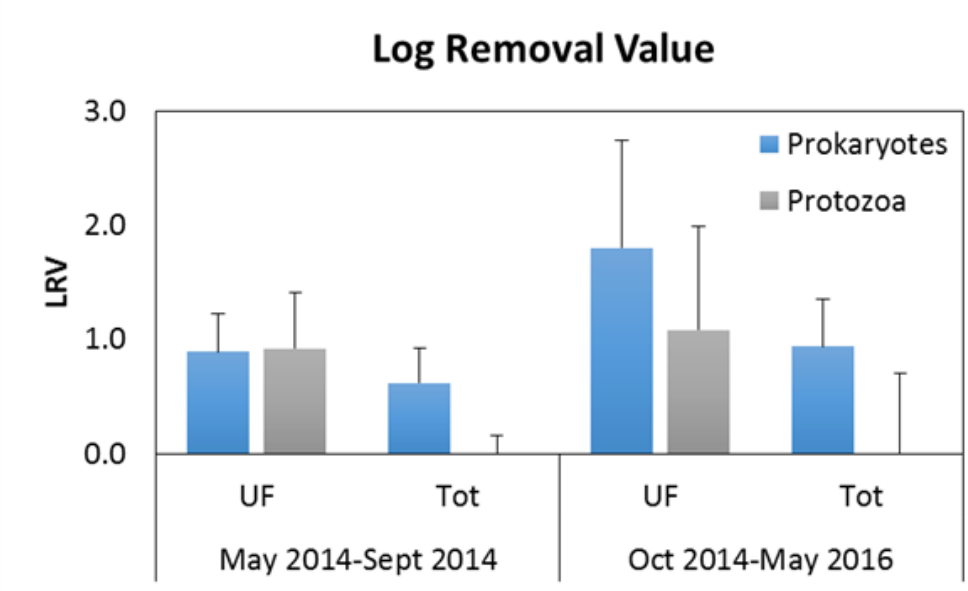


Figure 24 Log Removal Value (LRV) measured from abundance values of prokaryotes and protozoa in influent and effluent waters through the ultrafiltration unit (UF) and the complete treatment train (Tot).

In order to further characterize the aquatic prokaryotic community, which represented the most abundant component in all water samples, additional parameters were retrieved by cytometric analyses (Figure 25). The mean value of the forward scatter signals could provide an indication of the mean cell size patterns along the treatment train. Both sand filtration and ultrafiltration units significantly affected the mean size of prokaryotes, showing an overall reduction through SF unit but, unexpectedly, an overall significant increase through UF modules. The mean value of the green fluorescence signals is related to the per-cell content of nucleic acids. Thus, it could provide indications on the structural and metabolic state of the community. The mean fluorescence increased significantly when waters passed through the full-operating UF unit (Oct 2014-May 2016) (Table 5). The prokaryotic community was composed of two main sub-populations of cells having a different content of nucleic acid (namely, LNA and HNA cells with low and high nucleic acid content). The UF units were likely to modify the relative occurrence of these sub-populations (Table 5). Since HNA cells are generally bigger in size and brighter in fluorescence, the relative increase of HNA could explain the size and fluorescence variations of the prokaryotic community.

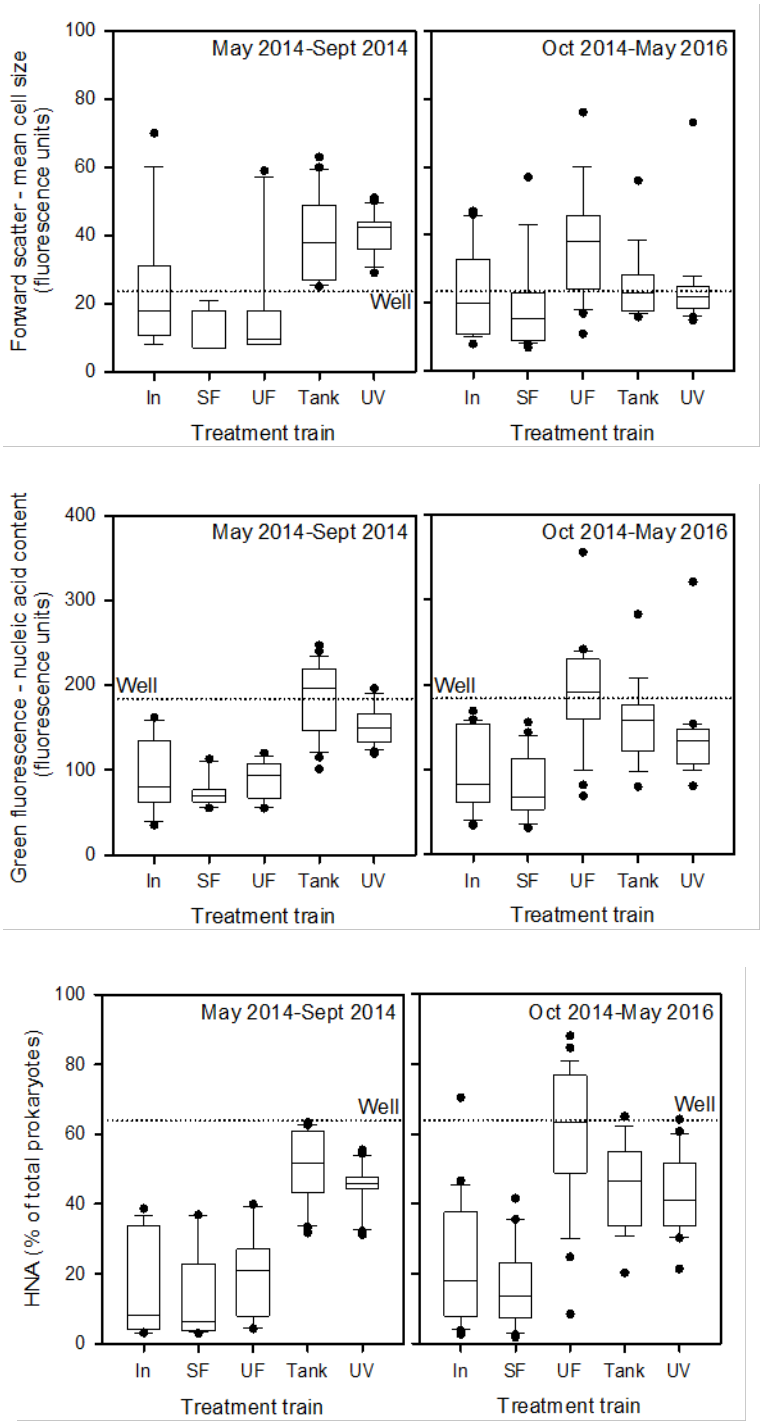


Figure 25 Box-plots of additional cytometric descriptors of the prokaryotic community assessed before and after replacement of the ultrafiltration modules.

Lines within the boxes, boundaries, whiskers and dots mark the median, 25th & 75th percentiles, 10t & 90th percentiles, and outliers, respectively. The Cleveland linear interpolation method was used to compute the percentile values. The dotted lines refers to the median value in well waters alternatively used for crop irrigation.

Table 5 Statistical comparison of samples collected along the treatment train (see Figure 25).

The p-values indicate the statistical significance of the difference in the median values between the two adjacent treatments. p<0.001 = ***; 0.001<p<0.01 = **; 0.01<p<0.05 = *; p>0.05 = no statistical difference.

Mann-Whitney U Statistic (p-value)		
Prok size	**May 2014-Sept 2014**	**Oct 2014-May 2016**
In vs SF	0.003*	0.322
SF vs UF	0.047**	<0.001***
UF vs Tank	<0.001***	0.018**
Tank vs UV	0.241	0.628
Prok fluo	**May 2014-Sept 2014**	**Oct 2014-May 2016**
In vs SF	0.184	0.222
SF vs UF	0.175	<0.001***
UF vs Tank	<0.001***	0.042*
Tank vs UV	0.037*	0.167
%HNA	**May 2014-Sept 2014**	**Oct 2014-May 2016**
In vs SF	0.340	0.248
SF vs UF	0.064	<0.001***
UF vs Tank	<0.001***	0.013**
Tank vs UV	0.058	0.525

The live/dead double staining analysis showed that the prokaryotic community was almost entirely composed by viable cells, since membrane-compromised cells (namely, dead cells) were generally far below 2% of the total cells. Interestingly, despite UV disinfection is not reported to produce detrimental effects on cell membranes, dead cells doubled after UV irradiation during both sampling periods and these increases were statistical significant (Figure 26). It could be a side effect from additional factors affecting the membrane integrity of a small fraction of the total community (e.g., local heat production, cell cluster disaggregation). However, this should be further evaluated by using different and more specific approaches (First & Drake, 2013).

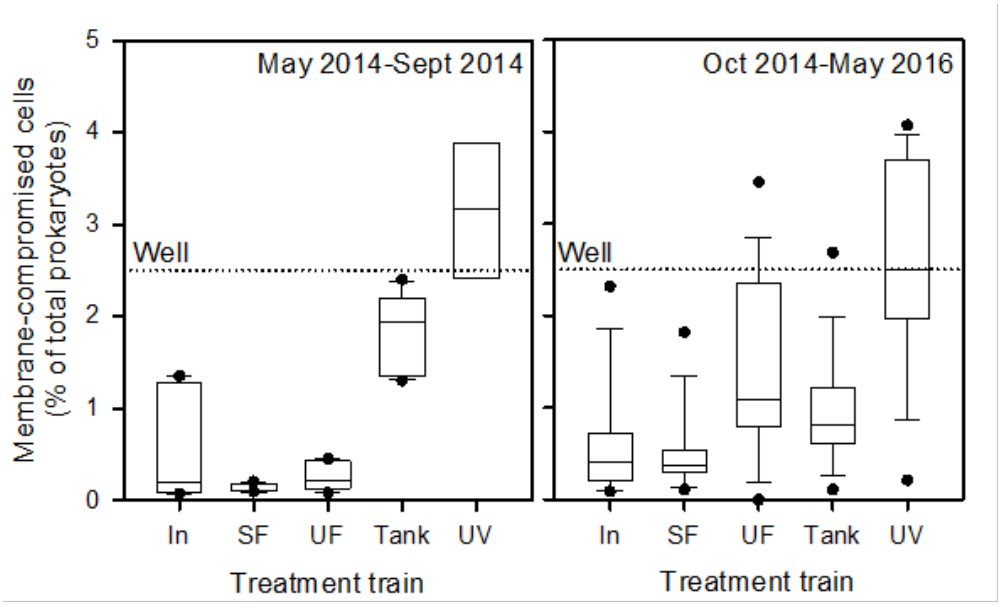


Figure 26 **Box-plots of membrane-compromised cells along the treatment train within the two sampling periods.**
Lines within the boxes, boundaries, whiskers and dots mark the median, 25th & 75th percentiles, 10t & 90th percentiles, and outliers, respectively. The Cleveland linear interpolation method was used to compute the percentile values. The dotted lines refers to the median value in well waters alternatively used for crop irrigation.

The patterns of the aquatic microbial components circulating along the treatment train were summarized by the principal component analysis (PCA). The PCA biplots of the two sampling periods showed similar patterns, with PC1 explaining most of the dataset variation (>75% of the overall variability). The main differences were found clearly on harsher effects of the full-operating UF modules on the prokaryotic community structure in comparison to the lowered operating conditions (Figure 27).

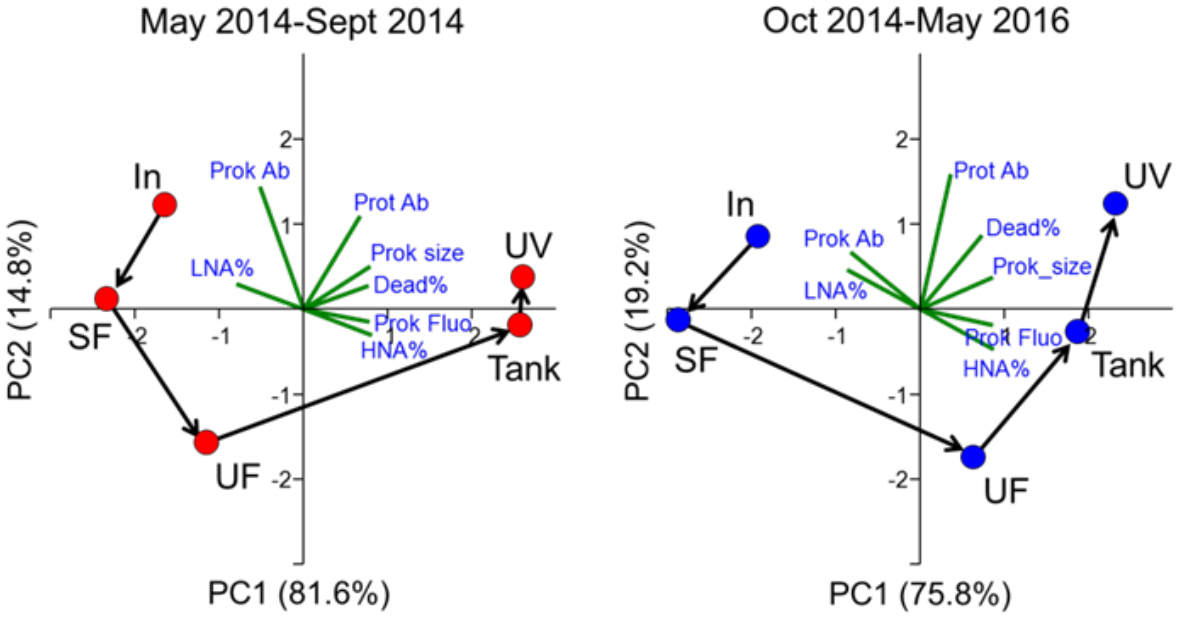


Figure 27 **Biplots of the PCA carried out via the data correlation matrix.**
Flow cytometric parameters were projected onto the factor space as active variables representing the structural features of the microbial community circulating through the tertiary treatment plant within the two sampling periods.

3.2.2 Faecal indicators

Initially, the results of the first monitoring campaigns, performed at the beginning of 2014, indicated the presence of faecal contamination (up to 1000 MPN/100mL of *Escherichia coli*) in the membranes permeate, suggesting a possible leakage. Then new modules were installed. After the substitution of the membranes, the tertiary treatments, composed of sand filtration, membrane ultrafiltration and UV radiation, allowed to comply with the standards related to *E.coli* (10 CFU/100mL for 80% of the samples), so providing between 3 and 5 logs removal (Figure 28). *Salmonella* was never isolated in any sample of water, soil and plant.

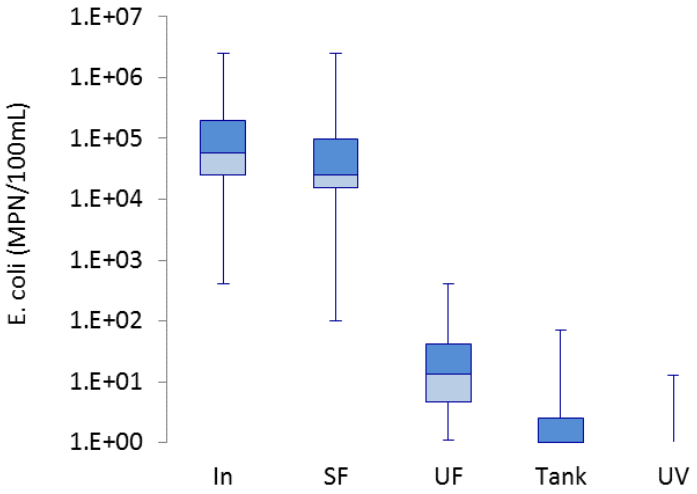


Figure 28 Average performances of the tertiary treatment during the period 20/10/2014-31/12/2015. Box plot of the concentrations of *E.coli*.
Sampling points: influent to the sand filter (In), sand filter outlet (SF), ultrafiltration membranes outlet (UF), storage tank (Tank), after UV disinfection (UV).

It is also important to highlight that two phenomena were observed in the tank where the tertiary treated wastewater was stored: (i) an increase of suspended solids concentration (up to 100%); (ii) a relevant die-off of the faecal indicator *E. coli*. In order to estimate the decay rate of *E. coli* in the storage tank, a sample of secondary treated wastewater was stored in a bottle at 20°C in the dark and results showed that *E. coli* concentration decreased by about 1 log after 5 days of storage (see Figure 29). The suspended solids increase can be associated to a bacterial re-growth, as shown by the results of the prokaryotic abundance, measured through Flow Cytometry analysis, which were displayed in Figure 23. The considerable extent of both bacterial re-growth and *E. coli* die-off was caused by the particular type of storage performed, which was static (meaning that when the tank was full the membrane permeate was sent directly to discharge without passing through the tank), and this may have favoured bacterial re-growth. To avoid this, a different strategy should be used for storing the water: the membrane permeate should continuously pass through the tank before being discharged.

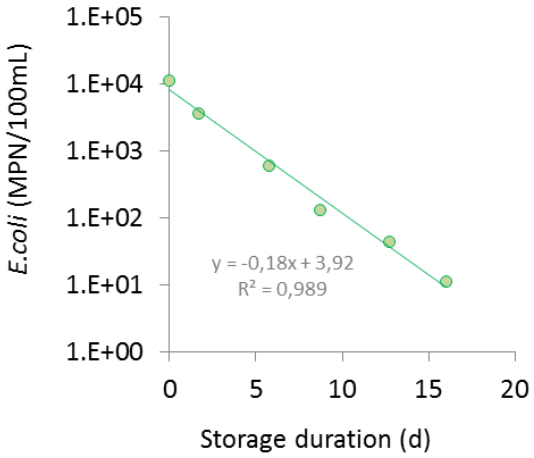


Figure 29 *E. coli* decay in a sample of secondary treated wastewater stored in a bottle at 20°C in the dark.

In terms of faecal contamination, the three types of water differed considerably. Compared with GW and TW, the non-disinfected SW was characterised by a considerably higher content of *E. coli*. This caused a significant presence of *E. coli* in the soil, but it did not result in any relevant contamination of plants or fruit (Figure 30). *E.coli* was never detected in fruits and only once in plants, which had been cultivated in a plot irrigated with conventional water (GW).

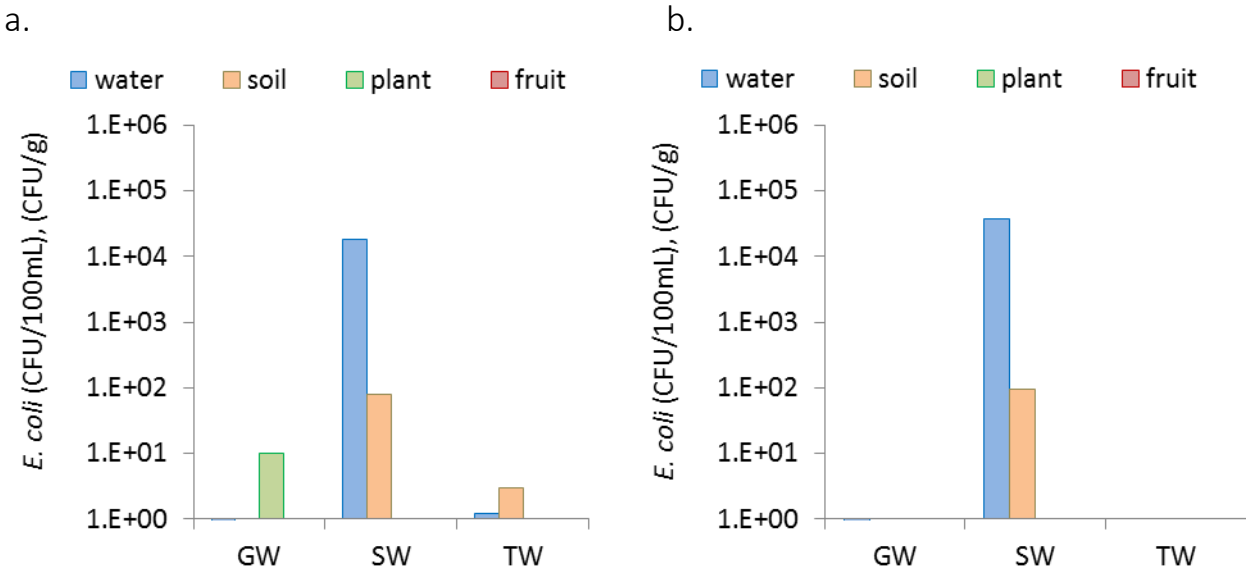


Figure 30 Presence of the indicator *E. coli* in the different irrigation water sources and in the corresponding irrigated soils and crops. Average values observed during the cultivation of tomato (a) and broccoli (b) are shown.

Faecal coliforms were also analysed in the different irrigation water sources and in the corresponding irrigated soils and crops. Results, displayed in Figure 31, confirm that the SW had a considerably higher faecal pollution than the other two water sources (on average, from two to three orders of magnitude more). However, irrigated soils and crops had similar content of faecal coliforms (differences were lower than one order of magnitude).

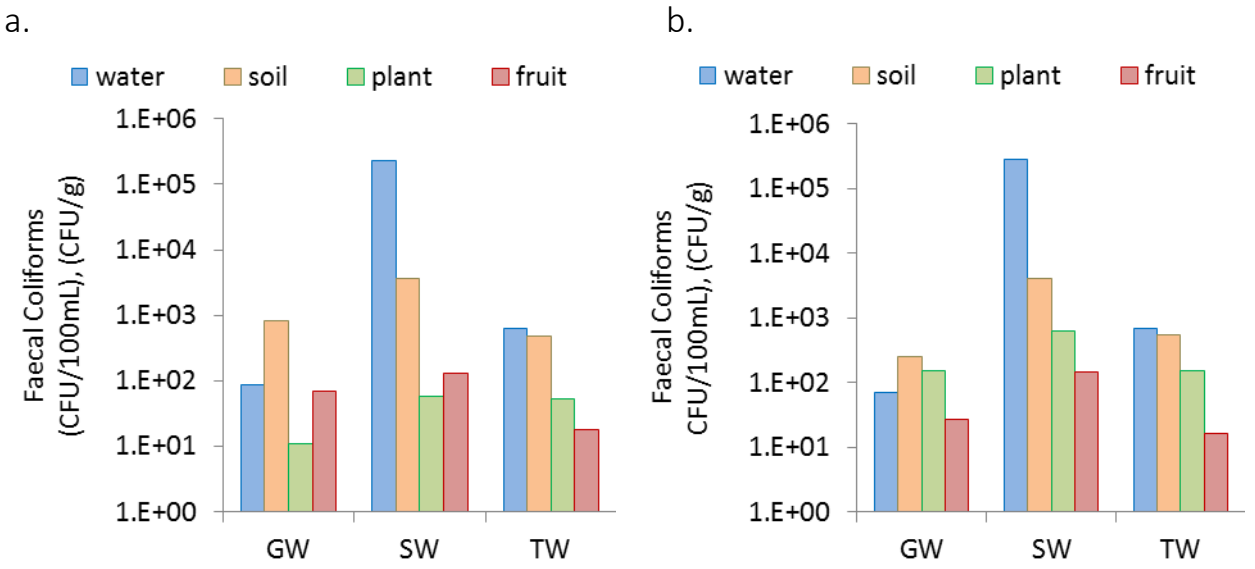


Figure 31 Presence of the indicator Faecal Coliforms in the different irrigation water sources and in the corresponding irrigated soils and crops. Average values observed during the cultivation of tomato (a) and broccoli (b) are shown.

Findings shown in Figure 30 and Figure 31 are in agreement with other studies (Cirelli et al. 2012; Palese et al. 2009) and indicate that irrigating with reclaimed secondary effluent having a residual presence of faecal contamination does not imply a relevant contamination of the crops. Moreover, the presence of *E. coli* on plants irrigated with well water (having no *E. coli*) suggests that, under the conditions applied in the present field study, the effect of possible external sources of contamination was comparable (or even higher) to the effect of contamination related to the irrigation water. Previous studies also highlighted the importance of the external environment, typically wildlife, as source of faecal contamination (Langholz & Jay-Russell 2013; Forslund, et al., 2012; Vergine, et al. 2015).

3.3 Molecular biological tools for the detection, identification and quantification of microbial contamination

3.3.1 Determination of indicator organisms

Following cultivation within selective media, *E.coli, Klebsiella* and Enterobacteriaceae were found in UV treated samples (Figure 32). Also in samples following performic acid treatment we have identified *E.coli* and *Enterococcus*, after ozone treatment additionally *Klebsiella* (Figure 32). Applying live-dead investigations on direct environmental samples (with no prior cultivation steps) we could identify an even broader range of bacteria – for instance *Shigella, Mycobacterium, Clostridium, Legionella, E.coli* and *Pseudomonas* in samples after performic acid treatment. Particularly *Mycobacterium* and *Clostridium* are very resistant organisms, due to their robust cell wall or spore-forming abilities. *Legionella* is a hygienically very relevant group and includes *Legionella pneumophila*, causing legionellosis, a form of pneumonia. These bacteria are of special relevance in assessing the risk of specific reuse strategies, because they can pose a severe risk, if aerosols are formed during the process. Recent outbreaks of legionellosis in Warstein (Germany) were also linked to a waste water treatment plant.

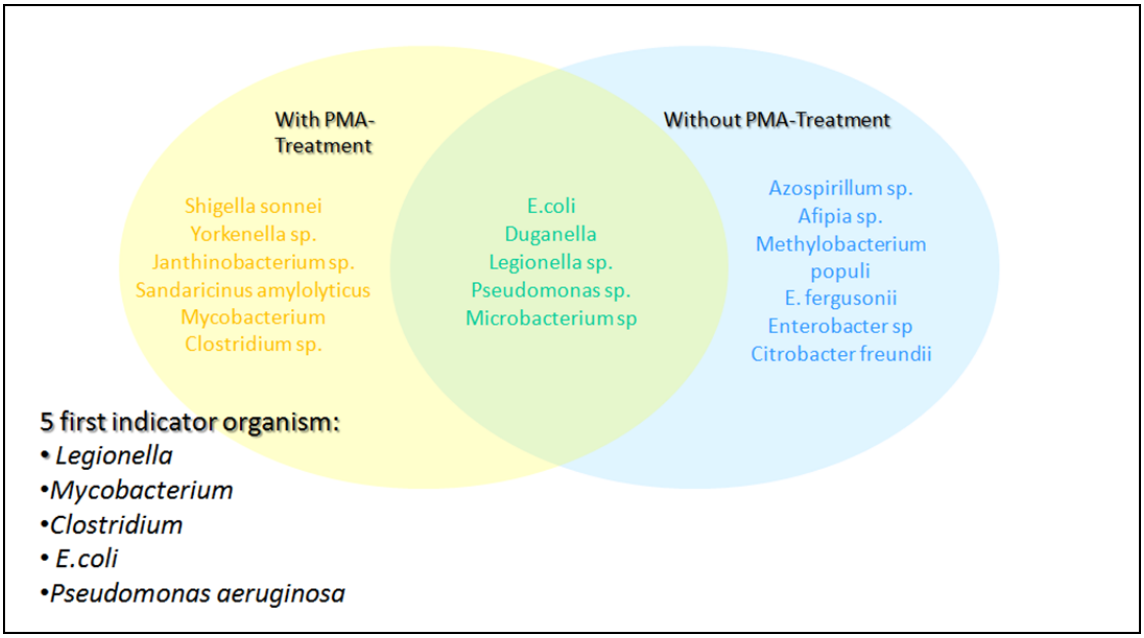


Figure 32 Results of cloning and sequencing of PMA treated and PMA untreated samples from effluent water.

Mycobacterium, Clostridium, Pseudomonas aeruginosa, *Escherichia coli* and *Legionella* have been chosen because they appeared to be both relevant and dominant in the viable population after treatment. We have chosen suitable primer sets for each organism and subsequently optimized the PCR with each primer set. However, some of the primer sets and amplification protocols will need further optimization and have been excluded in this study. For first tests we have used samples after performic acid and UV treatment as well as samples from different DEMOWARE project partners. Clostridium and Mycobacteria represent organisms, which are more resilient to pass through disinfection due to the ability to form spores (*Clostridium*) or a thick cell wall (*Mycobacterium*). *Pseudomonas spp*. are known to produce a high amount of extracellular matrix and form biofilms. As a result, the ability to form biofilms can protect the bacteria from disinfection. Slow diffusion of the disinfectant through the extracellular polymeric substance matrix also reduces the delivery of disinfectants to bacteria, which exist deeper in the biofilm (Stewart, PS, 2003). *Pseudomonas aeruginosa* acts as a representative for biofilm-forming organisms. *Legionella spp*. are also hygienically relevant, possibly pathogenic organisms and were included as an indicator organisms

Further results from deep sequencing analyses (Figure 32) provided a comprehensive insight into the whole population and allowed to determine the dominant as well as some of the background organisms in the population. Alongside the determined indicator organisms there are some additional organisms of interest, which could be detected in higher numbers after disinfection steps. For instance, *Flavobacterium* and *Aquabacterium* appeared distinctly increased after disinfection with peracetic acid. Both bacteria have an ability to adhere at surfaces and to form biofilms. Therefore they may survive disinfection better. Another example of bacteria which could be detected after disinfection with performic acid are *Arcobacter* and *Jiangella*. Besides its biofilm-forming ability, it was already reported that waste water treatment plants are an important reservoir of *Arcobacter*. There have been at least three documented drinking water outbreaks, associated with *Arcobacter*, which may qualify it as an additional interesting indicator organism. The genus *Jiangella* is related to Actinomycetes, which are able to form biofilms and show resistance to chlorine. Currently, we have no explanation for their increased occurrence in our samples. Development of indicator system (PMA, Testing, LED Incubator etc.). With all results it is important to

note, that within the confines of the project only a relatively limited set of samples could be analyzed and further studies may significantly improve the data situation.

During the project we tested specific primer set and qPCR DNA standards for the following organisms:

- Legionella spec.
- *Pseudomonas spec*
- *Mycobacteria*
- Clostridium
- *Escherichia coli*

In addition a universal bacterial primer set, which has been used to analyze the whole bacterial population, was tested with environmental samples.

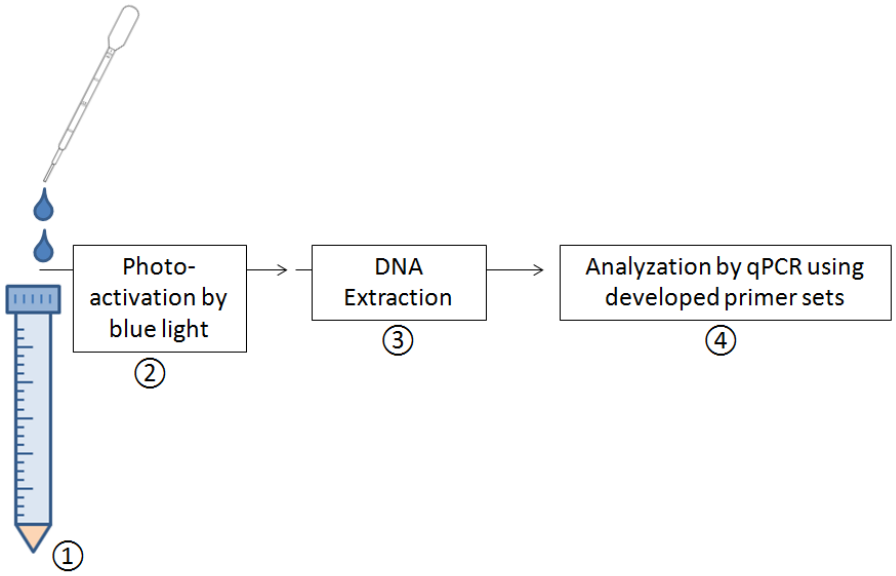


Figure 33 Workflow of the indicator system
① PMA-Treatment, ② photoactivation, ③ DNA extraction, ④ qPCR

The final workflow of the indicator system has been established within the project and is shown in Figure 33. Samples (up to 15 ml) are collected in a sterile sampling tube.

The samples or aliquots thereof are mixed with PMA to achieve a final concentration of 50 µM PMA. for the PMA treatment (Figure 33, ①). In order to allow for a comparison, samples before and after disinfection processes should be included. After a short incubation time (5 minutes), the sample will be irradiated with blue light (Figure 33, ②) for 15 minutes. There are commercial blue LED lamps, available for on-site laboratories (Geniul). In this study we tested a portable device, which was self-developed and emits a wavelength of 470 nm. Turbid samples should be diluted. Subsequently, the treated sample is stabilized and transported to the laboratory (for on-site sampling), where the DNA Extraction step can be performed (Figure 33, ③) and the qPCR System is applied to screen the samples for the predetermined indicator organisms (Figure 33, ④). The system can allow for a determination of pre-defined signal values for an insufficient disinfection (within 3-4 h) and potentially allows for a quicker intervention (risk management) than classic cultivation.

3.3.2 Evaluation of indicator system

To evaluate the developed PMA-qPCR system and to assess the applicability, results of samples, which were analysed by FCM and PMA-qPCR, were compared. Figure 34 shows different samples of the OFWRP and non-potable distribution network samples from before and after the treatment process (Figure 2), consisting of a hollow fibre membrane and chlorine disinfection. Samples were either treated or not treated with PMA. The left ordinate shows the FCM results in cells/mL, the right ordinate the qPCR-results in DNA copies/ mL, due to the use of universal bacterial primers which amplify most of the bacterial DNA in the sample. The results therefore, are not species-specific and qPCR-results could in this particular case only indicate DNA copies/mL and not the equivalent cells/mL as determined through FCM. In samples not treated with PMA (-PMA) DNA of both damaged and intact cells would be replicated, thus these counts can be related to the total cell counts in flow cytometry. On the contrary, in PMA treated samples (+PMA) only DNA of intact cells will be replicated which should correlate with the intact cell counts in FCM.

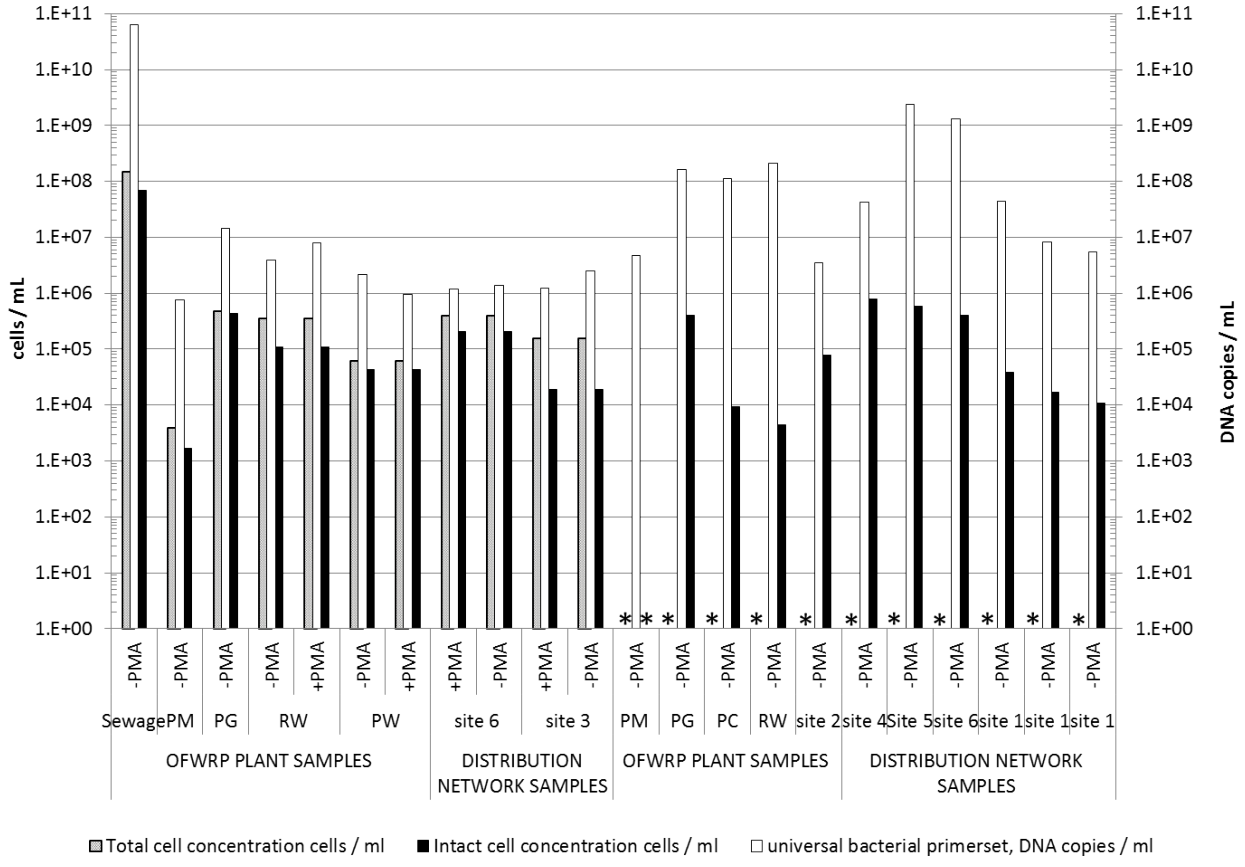


Figure 34 Comparison of FCM results and PMA-qPCR results for samples of the Old Ford Water Recycling Plant, UK
Where +PMA =samples treated with PMA, -PMA untreated samples, PM = post membrane, PG = post GAC, RW = reclaimed water and PW = potable water. Sites which were either not sampled or analysed are represented by *.

Determined qPCR results reveal the similar tendency in comparison to the determined FCM results: if the detected FCM-results decreased, the determined qPCR-results also decreased (Figure 34).

The FCM method allowed determination of the total cell concentration and distinguishes between membrane compromised and intact cells. It is possible that the analysis by live/dead FCM of bacteria, which produce a high number of extracellular matrix such as *Pseudomonas aeruginosa*, would be difficult (Zach-

arias et al, 2015). It also should be considered, that different division stages and various numbers of plasmids result in variable DNA content and therefore in different fluorescence intensities (Hallin et al, 2004, Cazalet et al, 2004). PMA-qPCR allows distinction between membrane compromised cells and intact cells by the use of the DNA-binding dye propidium monoazide, which diffuses into membrane compromised cells and in turn inhibits the following PCR steps.

To further evaluate the efficacy of the PMA-qPCR method it was tested on samples of treated waste water of the Ruhleben waste water treatment plant in Berlin, Germany. Samples were collected at three different days (sample 1, sample 2 and sample 3). Samples were treated with 4 ppm peracetic acid for 10 minutes. Sodium thiosulfate was used to neutralise the disinfectant. Afterwards, the non-disinfected and disinfected samples were analysed by both the PMA-qPCR method and cultivation with selective medium. Analyses were done in duplicate. To enumerate the total viable count, the samples were cultivated 48 hours with plate count agar at 22 °C and 36 °C degrees. The observed reduction brought about by the disinfection step is depicted in (Figure 35).

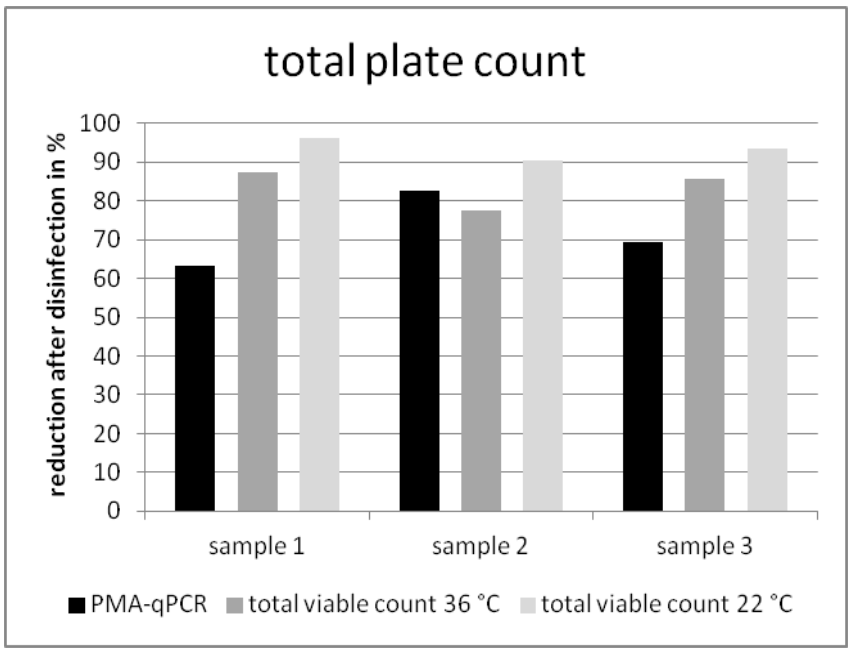


Figure 35 Comparison of cultivation (total viable count) and PMA-qPCR results (using bacterial universal primers). Averages of duplicate measurements

Reduction detected by the PMA-qPCR methods ranges between 63 % and 82 %. The values are higher (round about 85 to 95 %) when using the cultivation method. Comparing these two methods, it seems that the PMA-qPCR can indeed be used to monitor disinfection processes faster than with the regular cultivation based approach. However, a comparison of the results of the two tested methods is possible within limits only. In cultivation, only cultivable organism will be enumerated thus potentially underestimating the presence of bacteria. On the other hand also the analysis using qPCR, combined with a PMA-treatment, bears error sources. During the DNA-extraction step loss of bacterial DNA may occur leading to detection of lower gene copies numbers, too.

An additional validation trial for the PMA-qPCR method was done for developed primerset for *E. coli*. A pure culture of the lab strain *E.coli* K12 was heated at 95 °C for 20 min to kill the cells. After that, alive and dead cells were mixed in different ratios (100 % alive cells, 75 % alive cells, 50 % alive cells, 25 % alive cells and 0 % alive cells) and the prepared mixtures were analyzed by PMA-qPCR or cultivation with tryp-

tone soy agar at 37 °C. It is hypothesized that the alive cells detected by the two methods should correspond to the share in the prepared mixtures. Figure 36 displays the detected relation, where the percentage detected by the methods reflects the relative share compared to the value measured in samples containing 100% alive cells.

Samples with only heat-killed *E. coli* do not form colonies in the cultivation approach but still yield some results in the PMA-qPCR method (15 %). The match of applied and detected live cells is almost perfect for both methods at 25% share of alive cells. For samples containing 50 % or 75 % of alive cells results with both method deviate from the theoretical value, either over- or underestimating the viable cells. These results indicate that the PMA-qPCR for *E.coli* can be used to differentiate between alive and membrane-compromised cells with a comparable degree of confidence to the cultivation method, however, additional evaluations could be useful.

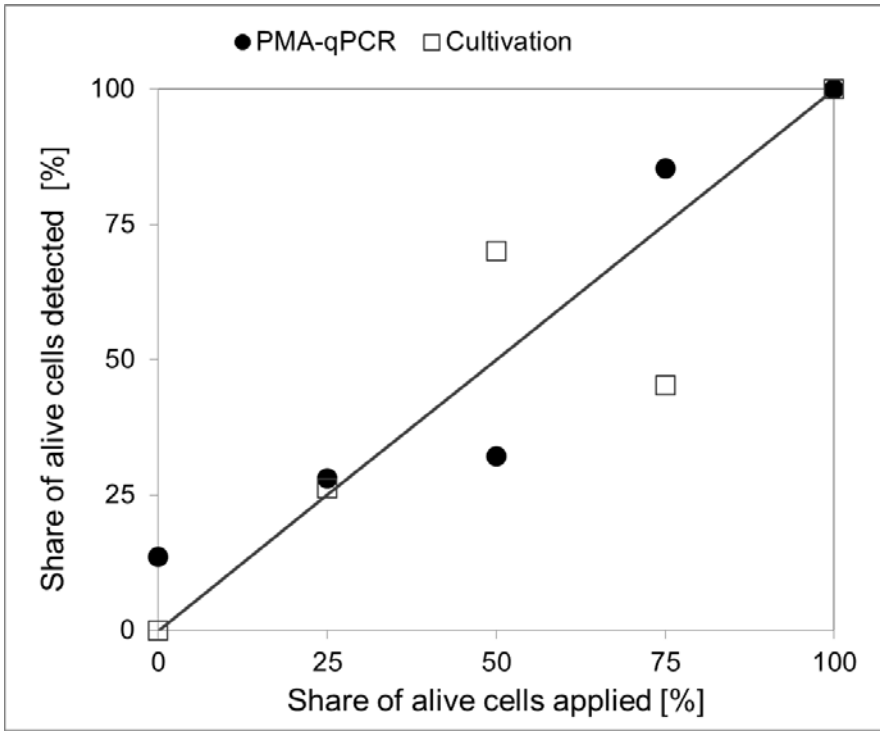


Figure 36 Comparison of cultivation (*E.coli*, cultivation by TSA) and PMA-qPCR results (using *E.coli* specific primers), the x-axis displays the theoretical percentage of live cells present in the sample, the y-axis displays the measured values in relation to the 100 % alive cell sample

In conclusion a first evaluation of the efficacy of the PMA-qPCR method is realised. The method appears to be a viable option to assess the effectiveness of disinfection processes. Trials with additional environmental samples will be performed after the project to further the commercialization of the indicator system.

Outlook to further applications of the PMA-qPCR method

As already mentioned, there are a lot of influences which should be considered: extracellular matrices of organisms would be a problem in flow cytometry. On the other hand, different division stages and particularly variable DNA content are important to consider when evaluating qPCR results in detail. Since the Propidium iodide-FCM method does not work with UV treated bacteria (intact membrane) we also investigated, if a PMA-qPCR is able to differentiate between UV treated and untreated cells of a spore forming

organism as well as of the indicator organism *E.coli* (data not shown). Interestingly, there were indications during the trials of a possible distinction between UV-treated cells and untreated cells of the spore-forming organism *Geobacillus stearothermophilu*s. While all samples were processed at the same day additional investigations are necessary which also take a closer look at the impact of the time between the UV treatment step and subsequent qPCR analyses.

PMA-qPCR can also be used to screen for specific bacteria, particularly in a mixed sample and is still relatively fast and sensitive. Unfortunately, the presence of inhibitors which might inhibit the qPCR, can lead to false negative signals, which can be mitigated by dilution of the samples as well as by use of an internal control. Without PMA, it is not possible to differentiate between live (membrane-compromised) and dead cells by qPCR and adjusting the performance and specificity of new primer sets remains challenging. Nevertheless, we demonstrated that the PMA-qPCR method can be used within limits (e.g. UV) to evaluate and monitor disinfection processes.

4 Overall discussion

The overall findings of this project have generated the answers to the proposed research questions in assessing FCM and qPCR, for identifying microbial contamination within water reuse applications including irrigation systems and black water reuse including non-potable distribution networks.

4.1 Applicability for assessing microbial contamination including limitations

The application of FCM to monitor the effectiveness of a multi-barrier treatment process of a blackwater reuse plant was found to achieve consistent results throughout the sampling period. Removal efficacies and microbiological community profiles were consistent with expectations with a 4 log reduction in total cell counts following UF and a >90% reduction in viable cells following chlorination. Results provide a baseline profile for the multi-barrier treatment process, enabling the identification of performance deterioration and maintenance requirements through significant deviations within these baseline values.

Similarly, the applicability of FCM was particularly useful for water reuse with irrigation applications, indicating that water quality was fundamentally affected by physical treatments and storage periods. Sand filtration and membrane UF showed very different efficiencies in regulating the occurrence of microorganisms and particulate debris. The sand filter was effective to reduce the TSS and some larger sized cells (e.g. protozoa and microbial aggregates), with most of particulate debris and microbial biomass transport largely unaffected. The ultrafiltration modules were more effective for removing both cell and particle densities, showing removal values of up to 2-logs.

With respect to conventional parameters monitored in water reuse schemes, FCM provides additional critical information in terms of spatial and temporal dynamics. However, conclusions with regards to the causes of system malfunctioning and inconsistent temporal variations could be erroneous when only the plant inflow/outflow waters are included in the sampling scheme. Similar outcomes were reported in studies focused on monitoring practices for drinking waters (Lautenschlager et al., 2013; Nescerecka et al., 2014).

Contrary to expectations, an increase in FCM counts was not observed during blackwater treatment for biologically dense processes (i.e. MLSS, RAS), with cell counts similar to those observed within the influent sewage. Extension of the gate to capture those particles with a higher fluorescence within the FL-1 and FL-3 channels did not significantly change the outcome. This is believed to be due to the aggregation of particles through the creations of flocs which may require further sample preparation prior to analysis. However, it is not anticipated that FCM should be widely utilised in characterising such dense biological processes (i.e. secondary/biological treatment) through, 1) the potential loss of biological material through sample preparation activities such as coarse filtering for the removal of large flocs etc.; 2) sensitivity of the equipment (clogging); and 3) restrictions around the limit of detection of the FCM. Manufacturers of different FCM models on the market report accurate analysis and quantitative FCM determinations in a range from 10^2 – 10^7 events/mL. Results throughout the FCM studies found cell concentrations beyond these ranges with the abundance of protozoa within irrigation applications below the detection limit in some cases, thus accurate estimations of LRV were potentially biased by cell abundance values close to the instrumental limits of detection.

The effects of disinfection processes, in particular chlorination can be evaluated through FCM and cell viability. Disinfection processes were found to not generate a great change in cell abundance, but resulted in a significant increase in the quantity of membrane-compromised cells in both irrigation and non-potable applications. The observed increase in compromised cells following UV treatment was not as

substantial as that observed following chlorination, with example compromised cell counts of > 90 % following chlorination in comparison to 4 % following UV. However, the mechanism of disinfection by UV treatment does not damage the cell-membrane identified through the double live/dead staining, with FCM analysis not representative of cell viability and UV efficacy. Additional and more specific FCM staining procedures should therefore be tested to assess UV specific effects on cell viability (First & Drake, 2013).

Initial results for PCR indicate that a monitoring of the success of disinfection treatments can be greatly enhanced by the application of molecular methods and can potentially lead to faster results than a cultural approach and can be applied to detect even non-cultivable hygienically relevant microorganisms. Unlike FCM, real-time PCR analysis was possible for highly enriched biofilm samples with the procedure not considered overly time-consuming. With the implementation of inhibition controls, the successful amplification of DNA can be verified. To quantify the amount of specific DNA-copies a custom primer set and standard DNA is needed for every organism of interest, but the approach (cloning and plasmid isolation) allows for easy extension of the spectrum of target microorganisms. The sample preparation can be standardized via existing DNA extraction protocols. In combination with the DNA stain PMA, it was possible to distinguish between viable and membrane-compromised cells. Real-time PCR with the PMA pretreatment step can thus serve as a fast and applicable tool for risk management.

4.2 Effectiveness of treatment processes based on surrogate parameters and maintenance regimes.

Whilst the devices and techniques evaluated during the course of the project have proven adequate for the analysis of microbial contamination within water reuse applications, surrogate parameters have been identified which correlate to the results (particularly for FCM) which can be utilised during operation as an indicator of performance and aid with maintenance activities. For example, enumeration of viable cells via FCM within the non-potable distribution network was found, as expected, to correlate to a free chlorine residual enabling the determination of a bespoke residual for the network to limit cell viability and regrowth. Conditions which resulted in the deterioration of the residual were identified (i.e. water age/stagnation) informing network maintenance activities and confirming the use of on-site chlorine monitoring during a network flush as a surrogate parameter to confirm effectiveness of maintenance. The evaluation of alternative parameters including carbon, nitrogen, phosphorus and TSS were not observed as potential surrogate parameters.

In the case of the irrigation application (as an example of a non-chlorinated process) a significant regrowth of cells owing to prolonged and varying storage periods in pipes and tanks, which are required to cope with irregular meteorological conditions and irrigation practices were observed. Unlike the non-potable distribution network an increase in suspended particulates were observed, probably owing to the detachment from pipe surfaces forced by pump waters outflowing from the UF modules. FCM resulted very useful in order to explain the causes of the suspended solids increase observed in storage tanks and as such used as a surrogate parameter to highlight the requirement of maintenance through flushing. The knowledge provided by FCM could be used in order to reduce the phenomena that caused the TSS non-compliance. At the same time, increased storage residence time results in reduced *E.coli*. However, when a final UV disinfection is performed in line with irrigation, it is preferable to avoid bacterial regrowth. Indeed, the suspended solids increase would limit the UV effectiveness, so reducing the overall disinfection performance.

Overall, the findings for FCM highlighted that a combination of surrogate parameters was essential for a complete description of the microbial dynamics along treatment trains and distribution networks. By

applying a high throughput technological approach to water monitoring practices, FCM provides a multiparametric dataset within minutes from sampling. It proved to be a suitable tool to monitor the fate and transport of both single-celled microorganisms (i.e., prokaryotes and protozoa) and particulate debris along the treatment train in a full-scale plant and on a long-term timescale (>1 year).

In relation to PCR, the current data is not sufficient to draw final conclusions about the effects of specific maintenance regimes or treatment processes on the population of chosen indicator microorganisms. It is however possible, to see a trend indicating not only a reduction of general organism count in all treated samples, but also a reduction of the specifically chosen indicator bacteria samples analysed with the newly developed indicator system. It is no surprise that a huge part of the viable population is reduced after UV and chemical treatments. The chosen qPCR method allowed for direct monitoring of critical and hygienically relevant portions of the populations. This is why the results of this study indicate an inhibited reduction of some of the hygienically relevant organisms. These findings are even more relevant with populations, which are part of a protected biofilm community. The tool now exists to allow for an easier assessment of specific maintenance and disinfection regimes and future results will help to understand the impact of specific treatments on the population dynamics of different wastewater treatment plants.

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