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Bacterial water quality and network hydraulic characteristics: a field study of a small, looped water distribution system using culture independent molecular methods.

Raju Sekar[1,2,3,a,b], Peter Deines[1,2,3,a,c], John Machell[1] A. Mark Osborn[3,d], Catherine A. Biggs[1,2] and Joby B. Boxall[1]

[1] Pennine Water Group, Department of Civil and Structural Engineering, The University of Sheffield, Mappin Street, Sheffield S1 3JD, UK

[2] ChELSI Institute, Pennine Water Group, Department of Chemical and Biological Engineering, The University of Sheffield, Mappin Street, Sheffield S1 3JD, UK

[3] Department of Animal and Plant Sciences, The University of Sheffield, Western Bank, Sheffield S10 2TN, UK

Corresponding Author:

Professor Joby B Boxall

Pennine Water Group, Department of Civil and Structural Engineering, The University of Sheffield, Mappin Street, Sheffield S1 3JD, U.K.

Phone: +44 (0) 114 2225760, Fax: +44 (0) 114 2225700 Email: j.b.boxall@sheffield.ac.uk

[a] These authors contributed equally to this research

[b] Present address: Department of Biological Sciences, Xi'an Jiaotong-Liverpool University, 111 Ren Ai Road, Dushu Lake Higher Education Town, Suzhou Industrial Park, Suzhou, Jiangsu, 215123, China

[c] Present address: Centre for Microbial Innovation, School of Biological Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand

[d] Present address: Department of Biological Sciences, University of Hull, Cottingham Road, Hull HU6 7RX, UK

ABSTRACT

Aims: To determine the spatial and temporal variability in the abundance, structure and composition of planktonic bacterial assemblages sampled from a small looped water distribution system and to interpret results with respect to hydraulic conditions.

Methods and Results: Water samples were collected from five sampling points, twice a day at 6 and 9 am on a Monday (following low weekend demand) and a Wednesday (higher midweek demand). All samples were fully compliant with current regulated parameter standards. This study did not show obvious changes in bacterial abundance (DAPI count) or community structure (DGGE analysis) with respect to sample site, hence to water age, however, the study did showed temporal variability with respect to both sampling day and sample time

Conclusions: Data suggests that variations in the bacterial assemblages may be associated with the local system hydraulics: the bacterial composition and numbers, over short durations, are governed by the interaction of the bulk water and the biofilm influenced by the hydraulic conditions.

Significance and Impact of the Study: This study demonstrates general stability in bacterial abundance, community structure and composition within the system studied. Trends and patterns supporting the transfer of idealised understanding to the real world were evident. Ultimately, such work will help to safeguard potable water quality, fundamental to public health.

INTRODUCTION

Although modern water treatment systems continuously provide high quality, safe drinking water, there are microorganisms that are resistant to inactivation or disinfection processes that survive and multiply within water distribution systems (WDS) (King *et al.* 1988;

LeChevallier *et al.* 1996; Szewzyk *et al.* 2000). There are also numerous theorised pathways by which contaminants, including microorganisms, can enter (Kirmeyer *et al.* 2001), colonise and multiply within WDS. Microbiological problems that have been associated with WDS include microbial regrowth (Camper, 2004; Regan *et al.* 2003), biofilm formation (Emtiazi *et al.* 2004; Berry *et al.* 2006), microbial corrosion (Beech and Sunner, 2004) and the presence of pathogens (Brettar and Höfle, 2008). Microbial regrowth and subsequent biofilm formation can increase microbial loads in WDS either by detachment of cells from biofilms (LeChevallier, 1990; Tokajian *et al.* 2005) and/or by acting as a reservoir (Mackay *et al.* 1998; Blanch *et al.* 2007). The factors that have been associated with regrowth of bacteria in WDS include temperature, water residence time (water age), concentration of organic compounds, residual disinfection concentration, and distribution system materials (Szewzyk *et al.* 2000; LeChevallier, 1990; Percival and Walker, 1999; Niquette *et al.* 2001; Keinänen *et al.* 2004). However, many of these studies used heavily idealised conditions and consequently practicable, predictive understanding of the behaviour and impact of microorganisms in real WDS remains limited. The aim of this study therefore, was to undertake field-based investigations into water quality, in particular planktonic bacterial water quality, to explore possible associations with local hydraulic conditions and water residence time.

Measurement of bacterial water quality

Regulatory assessment of microbiological loads of drinking water within WDS in the UK utilises culture-based approaches (APHA, 1989) typically utilising heterotrophic plate count (HPC)-based approaches (Sartory, 2004). However it is now established that typically less than 1% of the total number of bacterial cells that are present within environmental samples, such as drinking water, are culturable (Amann *et al.* 1995; Szewzyk *et al.* 2000).

Consequently, it is arguable and indeed likely that the widespread use of cultivation techniques limits our understanding of microbial assemblages present within WDS. For example, Burtscher *et al*. (2009) concluded that culture-based and culture-independent (e.g. using molecular) methods could reveal completely different results when applied to WDS. In particular they found that changes in the structure of bacterial communities, determined by culture-independent methods, corresponded to time of sampling, whereas in contrast, culture-based analyses revealed spatial associations of bacterial community structure within the distribution system. Culture-independent techniques (e.g. a direct cell counting technique in conjunction with a method that investigates community structure and composition) are however, becoming more common in studying microorganisms in WDS for research purposes, even if not for routine analysis (for example Zacheus and Martikainen, 1995; Schmeisser *et al.* 2003; Eichler *et al.* 2006; Obst and Schwarz, 2007; Kormas *et al.* 2009; Poitelon *et al.* 2009; Revetta *et al.* 2009; Deines *et al.* 2010). Such techniques can generate new understanding of bacterial behaviour within WDS, with the potential to help ensure public health.

Modelling water quality (and water age)

Potable water quality is generally defined in terms of physical, chemical and biological (including microbial) parameters. All these parameters vary spatial and as a function of time. The longer that water is left to react and interact within the WDS, the greater the potential for water quality change. This is due to reactions within the body of the water and at the interface with the pipe surface and ancillary fitting and fixtures. This fundamental association with time has led to the suggestion that ‘water age’ could be a useful first indicator or surrogate for water quality (USEPA, 2002).

Hydraulic network modelling software solves for flow velocities, which together with data on pipe lengths permits the determination of water residence times. These parameters, in combination with transport and tracking routines, enable the calculation of water age. Such functionality can be extended to model substance specific transformations. Rossman and Boulos (1996) present the modelling approaches commonly applied in WDS simulation software, while EPANET MSX represents the latest research development (Uber, 2009). However, application of substance specific water quality functionality is generally not practiced due to: incomplete understanding of the reactions and interactions occurring; uncertainty of algorithms; parameters and their values. Hence, water age remains a feasible and arguably potentially useful surrogate for water quality, but only an indicative surrogate.

Despite known limitations, such as model and parameter uncertainties, calibration methods and error propagation (Kang *et al*. (2009); Pasha and Lansey (2010) Boxall *et al.* 2004; Skipworth *et al.* 2002) water age is considered by many to be a potentially useful first indicator or surrogate for water quality. However there is a further complication with the concept of water age as applied in 1D hydraulic models. Most algorithms calculate water age as a flow weighted mean value of merged flows at a particular location (Machell *et al.* 2009). This is not intrinsically sensible for the surrogate of age, where the characteristics of mixed equal mass flow rates of 2- and 4-day old water are not necessarily those of 3-day-old water. Machell *et al*. (2009) suggested and developed an improved water modelling approach, which provides site-specific profiles of water age (including both mean and maximum) for an entire water distribution network. This modelling approach is used in this paper to determine the mean and maximum water age.

Impact of water age and hydraulic conditions on bacterial water quality

Previous studies have sought to explore the association between residence time and water quality, and microbial quality specifically, with variable results. While studying the effects of chlorine and residence time on the proportion (%) of culturable bacteria present either in biofilms or in bulk water, Srinivasan *et al*. (2008) found that as water residence times increased (8.2, 12, 24 and 48 h), the percentage of bacteria in the bulk water also increased (7, 37, 58 and 88%, respectively) in the presence of chlorine (0.2 mg l^{-1}). They also found that increasing concentrations of chlorine decreased the percentage of bacteria within the bulk water. Keinänen *et al*. (2004) studied the microbial community structure and biomass in developing biofilms in two drinking water distribution systems as a function of water residence time (ranging from 10 to 141 h). They showed that the microbial communities were rather stable through the distribution system, concluding that water residence time had only minor effects on the abundance of phospholipid fatty acids (PLFAs) (an estimate for viable microbial biomass). However, they also found that the viable bacterial biomass did increase with increasing water residence time in the WDS. Conversely Kerneis *et al*. (1995) found that water residence time, of up to 107 hours, did not have a significant influence on bacterial HPC densities within a WDS. However, they did find that bacterial HPC densities increased significantly with passage through and elevated residence times within reservoirs.

In addition to hydraulic conditions within a distribution system defining residence times, research has suggested that the local hydraulic conditions experienced by a given pipe will impact upon the water quality at that point in the system. Analyses of aesthetic factors such as discoloration, in both the field and laboratory, have shown the importance of pipe-specific hydraulic conditions for material build up and subsequent mobilisation (Boxall and Saul, 2005; Husband *et al.* 2008; Husband and Boxall, 2010).

It has also been recognized that hydraulic conditions play an important role in biofilm development and regrowth in water distribution systems (Pedersen, 1990; Kerr *et al.* 1999; Batté *et al.* 2003; Manuel *et al.* 2007). Such studies have primarily been conducted using bench-top reactor experiments, for example using increased rotational speed to examine biofilm 'sloughing' behaviour (Kerr *et al.* 1999; Manuel *et al.* 2007). While such work provides idealised understanding, the incomplete representation of real system behaviour limits application. LeChevallier *et al*. (1987) found high HPC in drinking water after opening taps that were turned off overnight, while Dreeszen (1997) reported favourable conditions for bacterial growth and attachment to the pipe surfaces in an automated watering system when the water usage and exchange rate was low. Increased biofilm formation in stagnant water, has also been reported (Barnes and Caskey, 2002; Tomaras *et al.* 2003). However, their studies were again conducted using culture-based approaches.

Research Questions

The aim of this research was to investigate spatial and temporal variability in planktonic bacterial abundance, community structure and the association with hydraulic conditions, in particular predicted water age, within a well defined, single pipe-material region of a WDS, where the water travels a relatively short distance but experiences significant water aging and mixing. The objective is to gain understanding of the inter-dependencies between bacterial abundance, community structure and composition with respect to varying water age and hydraulic conditions in a controlled field environment.

MATERIALS AND METHODS

Site description

This study focused on a confined, single pipe-material area within a WDS with a dominant user type providing defined demand patterns. The choice of system aimed to minimise, as far as possible, the uncertainties arising from system complexity and model calibration (in)accuracy. The WDS study site was identified in association with a collaborating UK water company and is shown in Figure 1. The site was selected to provide the required network simplicity, consisting of single pipe material (high pressure polyethylene - HPPE), of relatively short overall pipe length and consistent diameter, containing a single inlet but two nested loops to provide an interesting range of ageing and mixing effects. Internal pipe diameter from SP1 up to the turn towards SP2 is 144mm (~260m), all other pipes are 99mm internal diameter (~680m in total length). The system is supplied with a complex and variable blend of waters: upland with ferric sulphate, upland with alum, and river with alum floc based treatment trains. Importantly the site is in an area dominated by light industry providing a reliable and repeatable water usage pattern; with a well defined morning peak (on weekdays), reaching a maximum at 8 am, followed by lower, more constant day time use and very low night time and weekend demand. There is no permanent flow meter specifically associated with the study region, being part of a much larger District Metered Area (DMA). Temporary monitoring of the industrial users had previously been conducted, as part of field testing for hydraulic model build and checked with recent billing data, confirming flow pattern observations made here, this data is considered confidential and cannot be published.

Water age modelling

A recently built and pressure calibrated, 24 hour (weekday) hydraulic model in Aquis (7-Technologies, www.7t.dk/products/aquis/index.aspx) software was available for the WDS as a whole. This model was extensively checked against all available data (including the flow data noted above). It was not possible to conduct bespoke local hydraulic calibration

fieldwork. Fig. 2 shows the modelled flow rates in each pipe at peak hour (8am) and minimum hour (midnight) an order of magnitude change in flow rate can be seen in each pipe. The WDS model was run, using the functionality described in Machell *et al*. (2009), as a cycled 24 hour extended period simulation for 100 days to provide stable water age values. It should be noted that the model is for an idealised week day and does not include the effects of weekend usage. Hence the model is representative for both sampling days reported here, but not for the prior weekend period. Stable, repeatable mean and maximum age daily patterns were established in the region of interest after around 8 days; however, day 19 is reported herein, providing good confidence in model prediction stability without undue or excessive computation. The simulations used the main service reservoir supplying the WDS as the zero age reference, any complexities due to the hydraulic regime of the service reservoir are unknown and are not considered in the modelling. There are no other service reservoirs / storage facilities prior to the study region. Mean age results for 00:00 after 20 days of simulations are shown for the study region in Fig. 1. The calculated mean age at entry to the study region was 57 hours while the maximum was only slightly greater at 59 hours. As observed in the overall distribution system layout, this similarity in mean and maximum ages confirms minimal loops/mixing from the main service reservoir prior to the study region. However, it should be noted that the route is via a complex mix of pipe materials and conditions. To aid interpretation mean and max water ages are quoted hereafter as 24 hour average values of day 19 simulation results with values stated as relative to those at the entry to the study region (first sampling point).

Sampling locations

Five sampling points (SP) were identified within the network based on the water age calculations and mixing effects (Fig. 1). SP1 was located on the main entering the study

region, prior to the looped part of the system. SP2 and 3 are along the main flow route around the system, with no mixing effects. Fig. 3 shows the maximum age profile calculated at the start and end of the pipe length upstream of SP2, the pattern clearly shows the impact of the previously described working day demand. The flow route SP1 to SP2 to SP3 provides for exploration of ageing effects with no mixing. SP4 was located at a 'tidal' or 'balancing' point on one of the loops of the system, where flows meet from two directions. Depending on the exact time of day, model predictions were for the majority (~80%) of the water being younger, flowing via SP2, and the remainder being considerably older, flowing via SP3. SP5 was at the extremity of the study area, receiving the majority (~95%) of fairly young water directly from SP1 but also a small component having passed around much of the other loops. SP4 and SP5 were selected to provide interesting mixtures of water age. The mean and maximum ages for each SP relative to those at SP1 are given in Table 1.

Water sampling

Sampling for this investigation was undertaken at two time points on two days, 6am and 9am on Monday (day 1) and Wednesday (day 3). 6 am collection was intended to provide samples indicative of low flow conditions over night, while 9 am sampling was intended to capture the effects immediately after the morning peak in demand, such as mobilisation or sloughing of biofilm. Similarly, sampling on Monday was intended to investigate conditions after weekend low flow conditions (6 am sampling, in particular) while Wednesday would provide an indication of more stable weekday conditions. It should be noted that all sampling was of the bulk water and hence all data relates to the mobile, suspended phase. As with all buried, active, pressurised pipe systems there is no readily practicable available method to sample the biofilm itself.

In three sampling points (SP1, SP4, SP5) fire hydrants were available to collect water samples via a hydrant sampling tap. In SP3, the water samples were collected via a stand pipe attached to an industrial customer meter chamber (MSN) and in SP2 the samples were collected via a tap installed in a meter pit chamber (previously installed for demand monitoring, but not currently in use). The sample taps were flamed with a blow torch and water run through for 1 minute prior to collection of samples, following standard procedure for collecting drinking water samples (Rump, 1999). Ten litre water samples were collected in triplicate using plastic containers from each site at each time point. The samples were immediately stored in a mobile cooler, and returned to the laboratory within a maximum of 6 hours for further analysis, following the principles of MoDW Part 2 Sampling (the 'blue book'). This 6 hour constraint prohibited further sampling within a given day with the resources available to the project.

Standard water quality analysis

Physical chemical and biological (including HPC) analyses of the water samples were carried out on site or at the water company's accredited laboratory. Water temperature, pH, conductivity, total and free chlorine, iron, aluminium, and manganese concentrations and HPC were measured as per standard methods (APHA, 1989). In accordance with usual UK water industry practice no sample replicates were taken. This routine analysis did not include nutrient parameters such as phosphate or nitrogen species. Available data, not reported here for confidentiality reasons, from the associated treatment works and network as a whole are within regulatory limits and around UK average levels for the source water types.

Determination of bacterial water quality

In order to meet the main aim of this study, bacterial analysis beyond current regulatory driven practice was undertaken. Notably this analysis is based on culture-independent techniques.

Bacterial cell counts

For the direct counting method bacterial cell counts of the water samples were determined by staining the cells with fluorochrome 4',6-Diamidino-2-Phenylindole (DAPI) and imaging with an epifluorescence microscope (APHA, 1989). Fifty mL of water samples were fixed in 2% (v/v) of formalin for 15 hours. Fixed samples were filtered onto white polycarbonate membrane filters (pore size 0.22 µm, diameter 47 mm, Millipore Ltd., UK) and stored at -20 °C until further processing. The filters were cut into sections (ca. 1 cm cord length) and stained with DAPI solution (1 µg ml^{-1}), washed twice with distilled water and finally washed with 96% ethanol. Filter sections were then air-dried for ten minutes and examined using an Olympus DP71 epifluorescence microscope (Olympus, Essex, UK) equipped with a 100x oil immersion objective lens. The DAPI stained cells were visualized using a mercury vapour lamp and DAPI filter (U-MWU2- 330-385/420nm, excitation/emission) mounted on the microscope. The images were captured using CellB imaging software (Olympus Ltd., UK). A total of 780 fields of view were acquired from triplicate filter sections per sample and the DAPI stained cells were counted. From the image and membrane filter areas, the totals cells per filter was calculated and divided by the total volume of sample to determine the cells per mL of water. The variation in cell number with respect to sampling locations, time points and days were compared using univariate (1-way ANOVA) and multivariate (2-way ANOVA) analyses.

Bacterial community analysis using molecular techniques

294 *DNA extraction*

295 Two litres of each water sample were filtered onto white polycarbonate membrane filters
296 (diameter 47mm, pore size 0.22 µm, Millipore Ltd., UK) and stored at -80 °C until further
297 processing. DNA was extracted from the membrane filters by cutting half of a filter into
298 small pieces and placing them into the bead solution of the Ultraclean Soil DNA Isolation kit
299 (MO BIO, Carlsbad, U.S.A). DNA was extracted following the manufacturer's protocol and
300 eluted in 30 µl of nuclease free water and visualised on 1% (w/v) agarose gels.

301

302 *PCR amplification of 16S rRNA genes*

303 16S rRNA gene fragments were amplified by PCR using primers 338F and 530R (Whiteley
304 and Bailey, 2000). A GC-rich sequence (Muyzer *et al.* 1993) was attached to the 5' end of the
305 forward primer. Each PCR contained 5 µl of 10x PCR buffer, 10 µl of Q-Solution, 200 µM of
306 each of the dNTPs, 0.3 µM of each primer, 2.5 U of Taq DNA polymerase (Qiagen Ltd,
307 Crawley, UK) and 1 µl of template DNA and made up to a volume of 50 µl with sterile,
308 nuclease free water. An initial denaturation was carried out at 95 °C for 2 min, followed by
309 35 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min, and a final extension at
310 72 °C for 12 min. To confirm the amplification of DNA a positive control, *E. coli* MG1655,
311 was included. A negative (no-template) PCR control was used in order to ensure that there
312 was no DNA contamination.

313

314 *Denaturing gradient gel electrophoresis (DGGE) analysis*

315 DGGE analysis (Muyzer *et al.* 1993) was performed using the Bio-Rad DCode System (Bio-
316 Rad, Hercules, CA, USA). PCR-amplified DNA products (20 to 30 µL of product dependent
317 upon yield) were loaded onto 8% (w/v) polyacrylamide gels containing a denaturing gradient
318 ranging from 40 to 70% (80% denaturant consisted of 5.6 M urea and 32% (vol/vol)

deionised formamide buffer using 1xTAE for electrophoresis). Electrophoresis was performed at 100 V at 60 °C for 16 h. Due to the number of samples several DGGE gels were required to carry out the analysis. In order to normalise for differences between gels a PCR product from lab isolates was used as a marker on every gel (two outer lanes per gel). Following electrophoresis, gels were stained with SYBR-Gold (Invitrogen Ltd, Molecular Probes, Paisley, UK) for 45 min in the dark and scanned with an EpiChemi II Darkroom imager (UVP Inc, Upland, CA, USA). The DGGE band patterns were analyzed using Quantity One software (Bio-Rad). Bray-Curtis similarity matrices were calculated to compare the bacterial community patterns in different samples. Cluster analyses were done based on the presence of shared bands using the group average linking routine using Primer 6.0 software (Clarke and Gorley, 2006).

Excision and sequencing of DGGE bands

DGGE bands that were found commonly in all samples or the unique bands (found only in a few samples) were excised from the gel, via punching out of a gel piece using sterile pipette tips and then placed into 30 µl of sterile, nuclease free water and left overnight at 4 °C before further processing. The eluted DNA was used to re-amplify the DGGE band using the same primers (338F and 530R) as described above, except with no GC clamp. Each PCR contained 2 µl of eluted DNA prepared as mention above. An initial denaturing step of 95 °C for 2 min was followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, with a final extension step of 72 °C for 12 min. PCR products were verified by agarose gel electrophoresis along with DNA marker to confirm the length of amplified fragment. The PCR products were then purified using a QIAquick PCR purification kit (Qiagen Ltd, Crawley, UK) and eluted with 30 µl of sterile, nuclease free water. Purified bands were sequenced either with 338F or 530R primers using an ABI 3730 DNA analyzer (Applied

Biosystems, Foster city, CA). Sequences were edited and assembled using BioEdit Sequence Alignment Editor Program (Hall, 1999) and compared to GenBank using BLASTn to identify their closest related sequences (Altschul *et al.* 1990). DNA sequences were deposited in the GenBank database under accession numbers JQ268080 to JQ268091.

RESULTS

Standard water quality analysis

The range of physico-chemical and microbiological parameters measured using routinely applied standard methods across the five sampling locations during the study period are shown in Table 2. Turbidity and iron showed the greatest variation across the sampling locations. The turbidity values were higher at locations SP4 and SP5 (maximum values of 1.61 and 1.31 NTU, respectively, only ~40% of the current PCV [prescribed concentration value] of 4 NTU) when compared to the other three locations that had a maximum value of 0.29 NTU across all samples. Similarly, the iron concentration was higher at locations SP4 and SP5 (maximum concentration of 383 µg l^{-1} and 638 µg l^{-1}, respectively, unlike turbidity this is in excess of the current PCV despite being in a plastic pipe section of network) than at all other locations (maximum concentration of 188 µg l^{-1}, approaching the current PCV of 200 µg l^{-1}). There were nothing particular of note at SP4 and 5, such as the condition of the hydrant bowl, which would obviously have affected turbidity and iron levels. The free and total chlorine concentrations were low across the samples, with free chlorine around detection limits and total chlorine ranging from the detection limit to 0.15 mg l^{-1}. Although at low levels, there is a factor of four variation in total chlorine, lower values tending to occur at 6am at all sample points (6am average of 0.08mg l^{-1} with an standard deviation of 0.05 mg l^{-1}, 9am average of 0.12 mg l^{-1} with a standard deviation of 0.03 mg l^{-1}). This could indicate more reactions and interactions occurring overnight reducing the total chlorine in the water in the

6am samples.

It should be noted that with the exception of iron at SP4 and 5 all parameters, including the results for all microbial and indicator organisms, were within regulatory limits, indicating that this distribution system performs well from a WQ / regulatory perspective.

Bacterial water quality

Bacterial abundance (cell counts)

In general, the total bacterial count using the culture independent technique (DAPI) was 10^3 cells ml^{-1} higher in magnitude as compared to the 2 and 3 day HPC (Table 2) determined using standard methods. Mean bacterial cell counts were determined for each day (day 1 and day 3) and at each time point (6 am and 9 am) and at each sampling point across the study area with respect to the mean and maximum water age (Fig. 4). Despite the selection of a study region in which there was a significant increase in water age, differences in bacterial numbers at different sampling sites were not observed in relation to variation in the mean or maximum water age. Ignoring the more complex 'mixed age' sites (SP4 and 5), and considering only sites SP1, 2 and 3, there was no clear association between water age (mean or maximum) and bacterial numbers either across the different sampling days or times (Fig. 4a and b). Water samples collected from SP1 had a cell count ranging from 2.0 - 2.3 x 10^4 cells ml^{-1}, whereas samples from locations with high water age values, but no mixing (SP2 and 3) had mean bacterial numbers ranging from 1.9 - 2.6 x 10^4 cells ml^{-1} (Fig. 4a and b). The sampling locations SP4 and SP5 contained water with mixed ages (26-106 and 15-115 h, respectively) and bacterial numbers in those samples were in the range of 2.0 - 2.2 x 10^4 cells ml^{-1} and 1.6 - 2.7 x 10^4 cells ml^{-1}, respectively.

394 No obvious trend was detected with respect to sample site and hence water age, however
395 statistically significant but numerically small temporal variation (both day-to-day and
396 between samples taken at either 6am or 9am) was observed in bacterial abundance. Mean
397 bacterial numbers were higher on day 3 ($P<0.0001$) compared to day 1 at both 6 am and at 9
398 am (Fig. 4a and b). Mean bacterial numbers on day 3 ranged from 2.2 to 2.8 x 10^4 cells ml^{-1}
399 compared to 1.6 to 2.2 x 10^4 cells ml^{-1} on day 1. Mean bacterial numbers were also higher at
400 9 am compared to 6 am on both day 1 ($P<0.0001$; 1.2 to 1.9 x10^4 cells ml^{-1} at 6 am and 1.8 to
401 2.6x10^4 cells ml^{-1} at 9 am) and on day 3 ($P<0.05$; 2.0 to 2.7 x10^4 cells ml^{-1} at 6 am and 2.3 to
402 2.8 x10^4 cells ml^{-1} at 9 am). For the individual sampling locations, difference in bacterial
403 numbers between two time points on the same day were observed only in two locations (SP1
404 and SP5) and on day 1.
405
406 ***Bacterial community structure and composition***
407 DGGE analysis of water distribution samples at 6 am and 9 am on days 1 and 3 revealed a
408 highly consistent bacterial community structure (Fig. 5). The number of DGGE bands varied
409 between 10 and 13 depending on the sampling point and day (day 1: 10 bands, day 3: 13
410 bands). No marked differences were found between 6 am and 9 am sampling at any
411 individual location. Figure 6 shows that the clustering of the DGGE bands was primarily with
412 respect to sampling day, i.e. <70% similarity between communities sampled on day 1 and day
413 3, rather than to sampling time (6 am or 9 am) or location (SPs). The bacterial communities
414 within the majority of water samples collected on day 1 were >70% similar to each other
415 (Fig. 6, cluster I) whereas the communities in water samples collected on day 3 were
416 subdivided into two clusters (Fig. 6, clusters II and III) that shared 72% and 75%,
417 respectively, and additionally that these clusters revealed short-term temporal variability
418 between the bacterial communities sampled on Day 3. In contrast, the bacterial communities

sampled on day 1 did not show any differentiation with respect to clustering between 6 and 9 am (Fig. 6, cluster I).

The DGGE bands representing dominant and/or unique constituents of the community profiles were excised and sequenced (Table 3). Sequencing analysis (Table 3) showed that bacteria closely related to *Nevskia* sp. and *Escherichia* sp. were present at all sampling locations on all days and at all time points samples (Fig. 5). Sequences related to *Pseudomonas* sp. and *Bosea* spp. were found at all five sampling points, but were not identified at every site at every individual time point (Fig. 5). Sequences related to *Acinetobacter* spp. and another *Pseudomonas* sp. were found in only one sampling location (SP2) on day 1 (6 and 9 am) (Fig. 5).

DISCUSSION

Variation in bacterial abundance and community structure

The present study did not reveal a significant relationship between total bacterial numbers or community composition and sample location, and hence to either mean or maximum water age (residence time). This contrasts with a prior culture based (HPC) study of an experimental pipe rig (Srinivasan *et al.* 2008) that found an increasing percentage of bacteria in bulk water as water age increased. However, our findings are in agreement with those of Kerneis *et al*. (1995) who studied a water distribution network system, again via HPC analysis, and similarly found that water residence time did not have a significant influence on bacterial abundance. The lack of association with water age found here could be associated with the study region selected. The WDS in our study was comprised of single material, short

444 pipe lengths, but subject to significant residence times (Fig. 1 and Table 1). These
445 characteristics were selected to minimise uncertainty and complexity while providing
446 significant ageing and mixing effects. However, high residence times and short distances
447 indicate significant periods of low flow conditions. Such prolonged periods of low flow
448 conditions could have had a dominant influence on microbial abundance (Dreeszen, 1997;
449 Barnes and Caskey, 2002; Tomaras *et al.* 2003). In addition, the data reveals that the point of
450 entry to the study region (SP1) had nearly as great a variation in bacteria counts in the
451 mobile/suspended phase as occurred within the study region (SP2, 3, 4 and 5). This suggests
452 that upstream effects (such as the complex and variable mix of source water) could have
453 overshadowed net water quality changes during transit through the study area itself and/or
454 that daily or other cyclic behaviour within the area may have had a dominant effect upon
455 bacterial abundance.
456
457 In contrast to the lack of association with sample location and hence water age, both total
458 bacteria numbers and community structure showed correlation with sampling day and to a
459 lesser extent to sampling time (i.e. 6 am vs. 9 am; Figs. 4 and 6). Previously, Burtscher *et al.*
460 (2009) had shown temporal variability in bacterial community structure, albeit between
461 samples taken over one month apart. It is possible that the unique short-term changes in
462 bacterial abundance and community structure observed in our study reflect variability in the
463 hydraulic conditions. The study region exclusively serves light industry with well defined
464 daily demand patterns, predominately day time operation with minimal activity over the
465 weekend. On day 1 (Monday), following prolonged low flow over the weekend, lower
466 bacterial abundance and the presence of a distinct bacterial community was observed (Figs. 4
467 and 6), in comparison to day 3 (Wednesday). The difference in bacterial community structure
468 observed on day 3 and the corresponding increase in total bacterial numbers followed

exposure to 2 days of active hydraulic conditions. Cluster analysis (Fig. 6) also revealed clustering of communities on day 3, between communities sampled at 6 am, after night time low flow conditions, and at 9 am, after the morning peak in demand, and were again consistent to differences in bacterial abundance on day 3 at these time points. This pattern of increased bacterial numbers and of community clustering between 6 am and 9 am sampling was also observed on day 1, but to a lesser extent. It is apparent from the data that there is a greater reduction in total numbers of mobile / suspended bacteria following the long duration of low flow conditions at the weekend rather than overnight. Due to the long residence, or transport times, between sites these variations must be due to local effects rather than an artefact propagating through the system from upstream, such as overall variation in nutrient levels, background phosphate levels or other factors.

The cyclic behaviour of increasing and decreasing total numbers of bacteria and corresponding community clustering with inferred association to hydraulic conditions is in agreement with previous studies, particularly when considering the wider potential importance of interactions between biofilm and planktonic assemblages, such as biofilm sloughing. Our dataset shows that the total numbers of suspended / mobile bacteria are increased by hydraulically active conditions, agreeing with previous findings under idealised flow conditions (van der Wende *et al*. 1989; LeChevallier 1990; Kerr *et al.* 1999; Tokajian *et al*. 2005; Manuel *et al.* 2007). Previously this behaviour has been associated with biofilm detachment, sloughing or other mobilization, leading to the suggesting that this may be the case here as well. The reduced total numbers of suspended / mobile bacterial following low flow conditions (day 1) is also in accordance with an earlier study (Dreeszen, 1997) in which low water usage and exchange rate provided favourable conditions for bacterial growth and attachment to the pipe surfaces hence reducing mobile/suspended phases. Similar low flow

and enhanced biofilm growth was reported by LeChevallier *et al*. (1987); Barnes and Caskey, 2002; Tomaras (2003). In addition, our data set suggests that changes in bacterial community show some association with this cyclic variation in bacterial abundance, suggesting that the membership of the planktonic community is shifting between mobile and attached phases following low flow and active hydraulic conditions. This concept of cyclic behaviour in association with hydraulic patterns is also analogous, although opposite, to the iron corrosion processes observed by Smith *et al*. (1999) in which soluble iron concentrations increased in the bulk water during low flow periods and then declined due to flushing effects during demand hours due to corrosion processes. The suggested mobilisation of material from pipe walls by increased hydraulic conditions is supported by discolouration related work in live water distribution system (Boxall and Saul, 2005; Husband and Boxall, 2010).

Overall, these temporal patterns of bacterial abundance and community structure derived from field samples within an operational water distribution system can be inferred to suggest a complex quantitatively- and qualitatively-dynamic bacterial community influenced by attachment of cells to pipes during low flow periods and subsequent cell mobilisation into the suspended phase during active flowing periods. Moreover, such patterns will occur on a weekly and daily basis: with a weekly trend from prolonged low flows over the weekend promoting enhanced bacterial attachment, and a daily trend for weaker possibly more selective community attachment during night time low flow conditions that is readily mobilised or sloughed by each morning peak in water demand. Thus it seems that the bacterial composition, over short durations, is governed by the interaction of the bulk water and the biofilm. Direct sampling of the attached biofilm was not possible due to the buried and pressurised nature of active water distribution system. Further field sampling is required to fully substantiate these observation.

519

520 ***Composition of bacterial assemblages***

521 Most of the bacteria identified within the present study (*Acinetobacter, Nevskia, Escherichia,*
522 *Pseudomonas)* belonged to the Gammaproteobacteria except *Bosea* sp., which is an
523 Alphaproteobacteria. While studying changes in bacterial community within an urban water
524 distribution system in Greece, Kormas *et al.* (2009) found, via molecular analysis, that the
525 water samples collected from pumping wells and water treatment tank were dominated by
526 Betaproteobacteria whereas household waters were dominated by Actinobacteria. In the
527 present study, sequences closely related to *Nevskia* sp. and *Escherichia* sp. were found at all
528 sampling locations on both days and time points. Keinänen-Toivola *et al*. (2006) reported that
529 *Nevskia ramosa* was the dominant (53.8%) bacterial species present within a biofilm that
530 developed in a model drinking water system. The presence of *Nevskia* sp. and *N. ramosa* in
531 drinking water has also been reported in other studies (Revetta *et al.* 2009; Williams *et al.*
532 2004). Similarly, the presence of *E. coli* in drinking water and biofilm samples has been
533 previously reported (Blanch *et al.* 2007; Juhna *et al.* 2007; Liu *et al.* 2008). For example,
534 Juhna *et al*. (2007) detected *E. coli* in biofilms taken from drinking water distribution pipes
535 and coupons. It should be noted that there are numerous strains of *E. coli*, and that not all *E.*
536 *coli* are pathogenic. It should also be noted that these detections are by culture independent
537 method, so it is not clear whether the bacteria detected are viable or not and that no *E coli* or
538 coliforms were detected by culture dependent methods.

539

540 Sequences related to *Pseudomonas* spp. and *Bosea* spp. were found in all locations and on
541 both days, but not at every time point sampled (Table 3). Bacteria such as *E. coli* and
542 *Pseudomonas* spp. have been listed as commonly found species in drinking water, via HPC
543 analysis (Burtscher *et al.* 2009; Allen *et al.* 2004). The presence of *Bosea* sp. in drinking

water network has also been reported previously (Thomas *et al.* 2007) and members of this genus have been found to be resistant to decontamination procedures used for drinking water purification (Rapala *et al.* 2006). In the present study, sequences related to *Acinetobacter* were found in only one sampling location (SP2) on day 1 (6 and 9 am). The presence of *Acinetobacter* spp. has been previously reported in drinking water and in a water distribution network in Portugal (Simoes *et al.* 2007) and in Greece (Kormas *et al.* 2009), respectively.

Whilst this study has identified various bacteria as being present within the WDS sampled, it is recognized that the methods utilised herein have not demonstrated viability of particular species or quantified their prevalence. Treatment processes will physically remove *E. coli* and other bacterial species via clarification and filtration and many bacteria will be killed via chlorination (or other disinfection), hence bacteria present in treated water may not be viable. Hence, using the available techniques it is not possible to confidently determine whether living cells as opposed to inactivated or dead ones have been detected.

The system studied here is operated in a normal manner, is constructed of approved material and supplied with 'compliant' source water. Yet the culture-independent methods indicate three orders of magnitude more bacteria than using culture based methods. This is not cause for any action, compliance and public health risk is not compromised nor risk increased. The data demonstrates the complexity of the bacterial communities present in WDS. Greater future application of such techniques could help improve our understanding of the associations between bacteria, infrastructure and its condition, operation and maintenance practices and safeguarding public health. Interesting knowledge could be gained from consistent international comparison, for instance adding valuable information on the effects of disinfection regimes, such as different, and even no, chlorine residual.

Operational engineering overview

Cyclic patterns in bacteria numbers and in the structure of bacterial communities in WDS have been observed in this study and association with hydraulic conditions inferred. Sequence analysis has identified several bacterial species to be present within the system that have similarly been found within prior studies of other operational WDS and/or model WDS systems. It should also be noted that all regulated, physical, chemical (with the exception of iron) and microbiological parameters were within current PCV. From this it can be inferred that the presence of a diverse community of bacterial species within WDS is inevitable, but that the presence of bacteria does not necessarily constitute a health or other risk in itself.

System hydraulics are a factor that can be controlled and managed within WDS, through operational decisions, such as the control of flow routes through valve operations, and hence hydraulics can potentially be managed, such as through the use of network simulation and design tools (Prasad and Walters, 2006). Consequently, from the association between hydraulic conditions and the potential for bacterial attachment and mobilisation, suggested from our field data set and shown by previous studies under idealised bench top laboratory conditions, it may be possible in the future that the abundance, structure and composition of WDS bacterial communities could be indirectly controlled and managed. Understanding of the complex bacterial communities within WDS, and the environmental factors that control bacterial cell attachment and detachment, within real WDS could ultimately provide useful knowledge for systems management and further studies under field conditions are desirable.

Summary

Bacterial abundance, community structure and composition were studied for a short period in a small, well-defined region of a drinking water distribution system in England, UK. It should be noted that all sample results were fully compliant with current regulations (with the exception of iron samples at one site) and the microbial analysis generally demonstrates the stability in bacterial abundance, community structure and composition within the system.

Site-specific patterns of bacterial abundance and/or community structure were not observed within the pipe system, suggesting little association between bacterial assemblages and mean or maximum water age, however this was possibly masked by upstream variability.

When compared temporarily bacterial abundance varied between samples taken on Monday and Wednesday, and to a lesser extent with time of day, with corresponding temporal variability in community composition. Cyclic association with hydraulic conditions can be inferred, with both weekly and daily cycles.

This research suggests that a combination of hydraulic performance data and quantitative and qualitative molecular characterisation of WDS bacterial abundance, structure and composition can lead to a better understanding of the relationship between cell attachment and detachment processes and that this may be governed by hydraulic conditions. Such knowledge could prove valuable for WDS design, operation and management in the future.

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References

Allen, M.J., Edberg, S.C. and Reasoner, D.J. (2004) Heterotrophic plate count bacteria - what is their significance in drinking water? *Int J Food Microbiol* **92**, 265-274

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic Local Alignment Search Tool. *J Mol Biol* **215**, 403-410

Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identificaiton and *in situ* detection of indiviual microbial cells without cultivation. *Microbiol Rev* **59**, 143-169.

APHA (1989) *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, 17th Edition, Washington, D.C.

Barnes, R.L., and Caskey, D.K. (2002) Using ozone in the prevention of bacterial biofilm formation and scaling. *Water Conditioning and Purification*, Technical Report. October, p1-3.

Batte´, M., Appenzeller, B.M.R., Grandjean, D., Fass, S., Gauthier, V., Jorand, F., Mathieu, L., Boualam, M., Saby, S. and Block, J.-C. (2003) Biofilms in drinking water distribution systems. *Rev Environ Sci Biotech* **2**, 147–168.

Beech, I.B. and Sunner, J. (2004) Biocorrosion: towards understanding interactions between

biofilms and metals. *Curr Opin Biotech* **15**, 181-186.

Berry, D., Xi, C.W. and Raskin, L. (2006) Microbial ecology of drinking water distribution systems. *Curr Opin Biotech* **17**, 297-302.

Blanch, A.R., Galofre, B., Lucena, F., Terradillos, A., Vilanova, X. and Ribas, F. (2007) Characterization of bacterial coliform occurrences in different zones of a drinking water distribution system. *J Appl Microbiol* **102**, 711-721.

Boxall, J.B., Saul, A.J., and Skipworth, P.J. (2004) Modelling for hydraulic capacity. J Am Water Works Ass **96**, 161-169.

Boxall, J. B. and Saul, A. J. (2005) Modelling discolouration in potable water distribution systems. *J Environ Eng-ASCE* **131**, 716-725.

Brettar, I. and Höfle, M.G. (2008) Molecular assessment of bacterial pathogens - a contribution to drinking water safety. *Curr Opin Biotech* **19**, 274-280.

Burtscher, M.M., Zibuschka, F., Mach, R.L., Lindner, G. and Farnleitner, A.H. (2009) Heterotrophic plate count vs. *in situ* bacterial 16S rRNA gene amplicon profiles from drinking water reveal completely different communities with distinct spatial and temporal allocations in a distribution net. *Water SA* **35**, 495-504.

Camper, A.K. (2004) Involvement of humic substances in regrowth. *Int J Food Microbiol* **92**, 355-364.

Clarke, K.R. and Gorley, R.N. (2006) *PRIMER* v6: User Manual/Tutorial. PRIMER-E, Plymouth.

Deines, P., Sekar, R., Husband, P.S., Boxall, J.B., Osborn, A.M., and Biggs, C.A. (2010) A new coupon design for simultaneous analysis of in situ microbial biofilm formation and community structure in drinking water distribution systems. *Appl Microbiol Biot* **87**, 749-756.

Dreeszen, P.H. (1997) *Biofilms: Key to understanding and controlling bacterial growth in*

667 *automated drinking water systems*. Edstrom Industries Inc., Technical Report.
668 Eichler, S., Christen, R., Holtje, C., Westphal, P., Botel, J., Brettar, I., Mehling, A. and Hofle,
669 M.G. (2006) Composition and dynamics of bacterial communities of a drinking water
670 supply system as assessed by RNA- and DNA-based 16S rRNA gene fingerprinting.
671 *Appl Environ Microb* **72**, 1858-1872.
672 Emtiazi, F., Schwartz, T., Marten, S.M., Krolla-Sidenstein, P. and Obst, U. (2004)
673 Investigation of natural biofilms formed during the production of drinking water from
674 surface water embankment filtration. *Water Res* **38**, 1197-1206.
675 Hall, T.A. (1999) Bioedit: a user-friendly biological sequence alignment editor and analysis
676 program for Windows 95/98/NT. *Nucleic Acids Sym Ser* **41**, 95.
677 Husband, P. S., Boxall, J. B. and Saul, A. J. (2008). Laboratory studies investigating the
678 processes leading to discolouration in water distribution networks. *Water Res* **42**, 4309-
679 4318.
680 Husband, P.S. and Boxall J.B. (2010) 'Field Studies of Discolouration in Water Distribution
681 Systems: Model Verification and Practical Implications' *J Water Res Pl ASCE* **136**, 1,
682 86-94
683 Juhna, T., Birzniece, D., Larsson, S., Zulenkovs, D., Sharipo, A., Azevedo, N.F., Menard-
684 Szczebara, F., Castagnet, S., Feliers, C. and Keevil, C.W. (2007) Detection of
685 *Escherichia coli* in biofilms from pipe samples and coupons in drinking water
686 distribution networks. *Appl Environ Microb* **73**, 7456-7464.
687 Kang, D.S., Pasha, M.F.K. and Lansey, K. (2009) Approximate methods for uncertainty
688 analysis of water distribution systems. *Urban Water J* **6**, 233-249.
689 Keinänen, M.M., Martikainen, P.J. and Kontro, M.H. (2004) Microbial community structure
690 and biomass in developing drinking water biofilms. Can J Microbiol **50**, 183-191.
691 Keinnen-Toivola, M.M., Revetta, R.P. and Santo Domingo, J.W. (2006) Identification of

692 active bacterial communities in a model drinking water biofilm system using 16S
693 rRNA-based clone libraries. *FEMS Microbiol Lett* **257**, 182-188.
694 Kerr, C.J., Osborn, K.S., Roboson, G.D. and Handley, P.S. (1999) The relationship between
695 pipe material and biofilm formation in a laboratory model system. *J Appl Microbiol* **85**,
696 29–38.
697 Kerneis, A., Nakache, F., Deguin, A. and Feinberg, M. (1995) The effects of water residence
698 time on the biological quality in a distribution network. *Water Res* **29**, 1719-1727.
699 King, C.H., Shotts, E.B., Wooley, R.E. and Porter, K.G. (1988). Survival of coliforms and
700 bacterial pathogens within protozoa during chlorination. *Appl Environ Microb* **54**,
701 3023-3033.
702 Kirmeyer, M.M., Friedman, K. Martel, D., Howie, M. and LeChevallier, M. (2001) *Pathogen*
703 *intrusion into distribution systems.* AWWA Research Foundation project No. 436,
704 Denver, USA
705 Kormas, K.A., Neofitou, C., Pachiadaki, M. and Koufostathi, E. (2009) Changes of the
706 bacterial assemblages throughout an urban drinking water distribution system. Environ
707 Monit Assess **165**, 27-38.
708 LeChevallier, M.W., Babcock, T.M. and Lee, R.G. (1987) Examination and characterization
709 of distribution systems biofilms. *Appl Environ Microb* **53**, 2714-2724.
710 LeChevallier, M.W., (1990) Coliform regrowth in drinking water - a review. *J Am Water*
711 *Works Ass* **82**, 74-86.
712 LeChevallier, M.W., Welch, N.J. and Smith, D.B. (1996) Full-scale studies of factors related
713 to coliform regrowth in drinking water. *Appl Environ Microb* **62**, 2201-2211.
714 Liu, Y.M., Gilchrist, A., Zhang, J. and Li, X.F. (2008) Detection of viable but nonculturable
715 *Escherichia coli* O157 : H7 bacteria in drinking water and river water. Appl Environ
716 Microb 74, 1502-1507.

717 Mackay, W.G., Gribbon, L.T., Barer, M.R. and Reid, D.C. (1998) Biofilms in drinking water
718 systems - A possible reservoir for *Helicobacter pylori*. *Water Sci Technol* **38**, 181-185.
719 Machell, J., Boxall, J., Saul, A. and Bramley, D. (2009) Improved representation of water age
720 in distribution networks to inform water quality. *J Water Res Pl -ASCE* **135**, 382-391.
721 Manuel, C.M., Nunes, O.C. and Melo, L.F. (2007) Dynamics of drinking water biofilm in
722 flow/non-flow conditions. *Water Res* **41**, 551-562.
723 Muyzer, G., Dewaal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial
724 populations by denaturing gradient gel electrophoresis analysis of polymerase chain
725 reaction amplified glenes coding for 16S ribosomal RNA. *Appl Environ Microb* **59**,
726 695-700.
727 Nichols, G., Lane, C., Asgari, N., Verlander, N.Q. and Charlett, A. (2009) Rainfall and
728 outbreaks of drinking water related disease and in England and Wales. J Water Health
729 **7**:1-8.
730 Niquette, P., Servais, P. and Savoir, R. (2001) Bacterial dynamics in the drinking water
731 distribution system of Brussels. Water Res **35**, 675-682.
732 Obst, U. and Schwartz, T. (2007) Microbial characteristics of water distribution: Compiled
733 investigations in a German drinking water distribution system. Practice periodical of
734 Hazardous, Toxic and Radioactive Waste Management 11, 78.
735 Pasha, M.F.K. and Lansey, K. (2010) Effect of parameter uncertainty on water quality
736 predictions in distribution systems-case study. J Hydroinform **12**, 1-21.
737 Pedersen, K. (1990) Biofilm development on stainless steel and PVC surfaces in drinking
738 water. *Water Res* **24**, 239-243.
739 Percival, S.L. and Walker, J.T. (1999) Potable water and biofilms: a review of the public
740 health implications. *Biofouling* **14**, 99-115.
741 Poitelon, J.B., Joyeux, M., Welte, B., Duguet, J.P., Prestel, E., Lespinet, O. and Dubow, M.S.

(2009) Assessment of phylogenetic diversity of bacterial microflora in drinking water using serial analysis of ribosomal sequence tags. *Water Res* **43**, 4197-4206.

Prasad, T.D. and Walters, G.A. (2006) Minimizing residence times by rerouting flows to improve water quality in distribution networks. Eng Optimiz **38**, 923-939.

Rapala, J., Niemela, M., Berg, K.A., Lepisto, L. and Lahti, K. (2006) Removal of cyanobacteria, cyanotoxins, heterotrophic bacteria and endotoxins at an operating surface water treatment plant. *Water Sc Technol* **54**, 23-28.

Regan, J.M., Harrington, G.W., Baribeau, H., De Leon, R. and Noguera, D.R. (2003) Diversity of nitrifying bacteria in full-scale chloraminated distribution systems. *Water Res* **37**, 197-205.

Revetta, R.P., Pemberton, A., Lamendella, R., Iker, B. and Domingo, J.W.S. (2009) Identification of bacterial populations in drinking water using 16S rRNA-based sequence analyses. *Water Res* **44**, 1353-1360.

Richardson, H.Y., Nichols, G., Lane,C,, Lake, I.R. and Hunter, P.R. (2009) Microbiological surveillance of private water supplies in England: the impact of environmental and climate factors on water quality. *Water Res* **43**, 2159-2168.

Rossman, L. A. and Boulos, P. F. (1996) Numerical methods for modelling water quality in distribution systems: A comparison. *J Water Res Pl-ASCE* **122**, 137–146.

Rump, H.H. (1999) *Laboratory manual for the examination of water, waste water and soil.* Wiley-VCH, Verlag GmbH, Weinheim, Germany.

Sartory, D.P. (2004) Heterotrophic plate count monitoring of treated drinking water in the UK: a useful operational tool. *Int J Food Microbiol* **92**, 297-306.

Schmeisser, C., Stockigt, C., Raasch, C., Wingender, J., Timmis, K.N., Wenderoth, D.F., Flemming, H.C., Liesegang, H., Schmitz, R.A., Jaeger, K.E. and Streit, W.R. (2003) Metagenome survey of biofilms in drinking-water networks. Appl Environ Microb **69**,

767 7298-7309.
768 September, S.M., Els, F.A., Venter, S.N. and Brözel, V.S. (2007) Prevalence of bacterial
769 pathogens in biofilms of drinking water distribution systems. J Water Health **5**, 219–
770 227.
771 Skipworth, P.J., Machell, J. and Saul, A.J. (2002) Empirical travel time estimation in a
772 distribution network. *Proceedings of the Institution of Civil Engineers-Water and*
773 *Maritime Engineering* **154**, 41-49.
774 Silva, M.E.Z.D., Filho, I.C., Endo, E.H., Nakamura, C.V., Ueda-Nakamura, T. and Filho,
775 B.P.D. (2008) Characterisation of potential virulence markers in *Pseudomonas*
776 *aeruginosa* isolated from drinking water. *Antonie Van Leeuwenhoek* **93**, 323-334.
777 Simoes, L.C., Simoes, M., Oliveira, R. and Vieira, M.J. (2007) Potential of the adhesion of
778 bacteria isolated from drinking water to materials. *J Basic Microb* **47**, 174-183.
779 Smith, S. E., Holt, D. M., Delanoue, A., Colbourne, J. S., Chamberlain, A. H. L. and Lloyd,
780 B. J. (1999) A pipeline testing facility for the examination of pipe wall deposits and
781 red-water events in drinking water. *Water Environ J* **13**, 7-15.
782 Srinivasan, S., Harrington, G.W., Xagoraraki, I. and Goel, R. (2008) Factors affecting bulk to
783 total bacteria ratio in drinking water distribution systems. *Water Res* **42**, 3393-3404.
784 Szewzyk, U., Szewzyk, R., Manz, W. and Schleifer, K.H. (2000) Microbiological safety of
785 drinking water. *Annu Rev Microbiol* **54**, 81-127.
786 Thomas, V., Casson, N. and Greub, G. (2007) New *Afipia* and *Bosea* strains isolated from
787 various water sources by amoebal co-culture. Syst Appl Microbiol **30**, 572-579.
788 Tokajian, S.T., Hashwa, F.A., Hancock, I.C. and Zalloua, P.A. (2005) Phylogenetic
789 assessment of heterotrophic bacteria from a water distribution system using 16S rDNA
790 sequencing. Can J Microbiol **51**, 325-335.
791 Tomaras, A.P., Dorsey, C.W., Edelmann, R.E. and Actis, L.A. (2003) Attachment to and

biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology* **149**, 3473-3484.

Uber, J. G. (2009) Multi-species network water quality modelling: Current examples, future potential and research needs. key note paper CCWI 2009 in Integrating Water Systems, Boxall and Maksimovic (eds), Taylor and Frascis, London, pp 13-19.

USEPA (2002) Effects of water age on distribution system water quality. Prepared by AWWA with assistance from Economic and Engineering Services, Inc.http://www.epa.gov/ogwdw000/disinfection/tcr/pdfs/whitepaper_tcr_waterdistribution.pdf [accessed 9/8/2010]

van der Wende, E., Characklis, W.G. and Smith, D.B. (1989) Biofilms and bacterial drinking water quality. *Water Res* **23**, 1313–1322

Whiteley, A.S. and Bailey, M.J. (2000) Bacterial community structure and physiological state within an industrial phenol bioremediation system. Appl Environ Microb **66**, 2400-2407.

Williams, M.M., Domingo, J.W.S., Meckes, M.C., Kelty, C.A. and Rochon, H.S. (2004) Phylogenetic diversity of drinking water bacteria in a distribution system simulator. J Appl Microbiol **96**, 954-964.

Zacheus, O.M. and Martikainen, P.J. (1995) Occurrence of heterotrophic bacteria and fungi in cold and hot water distribution systems using water of different quality. Can J Microbiol **41**, 1088-1094.

Figures

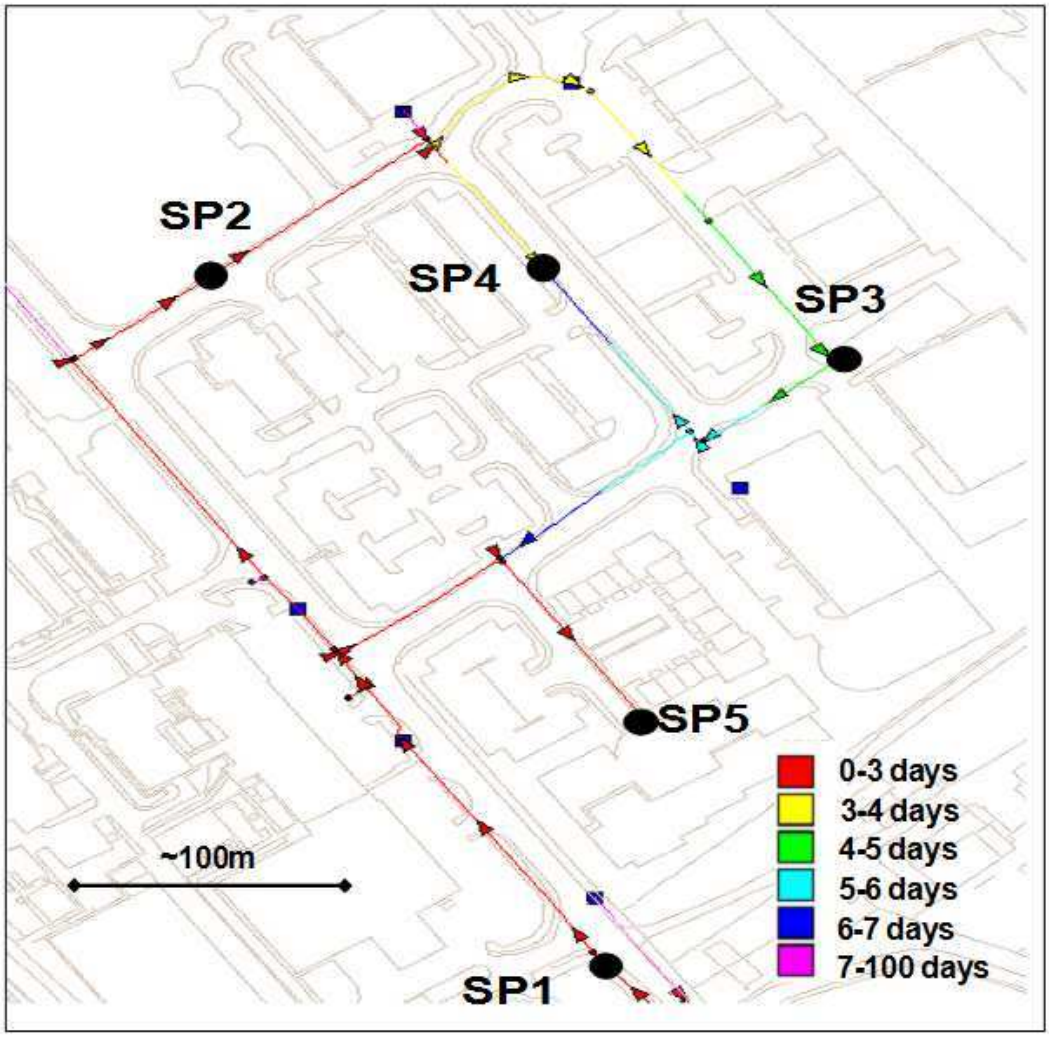


Fig. 1 – Schematic of study region showing sample points (SP), flow direction (arrows), pipes colour coded for mean water age and backdrop of the region.

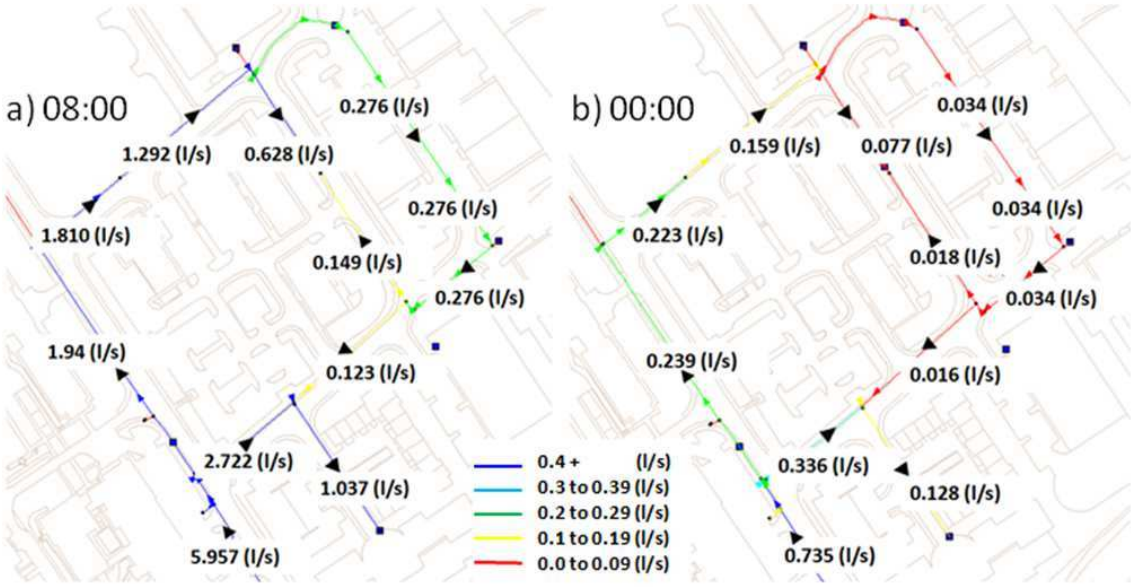


Fig. 2 - Network layout showing modelled pipe flow rates at a) high flow, 08:00 and b) low flow, 00:00

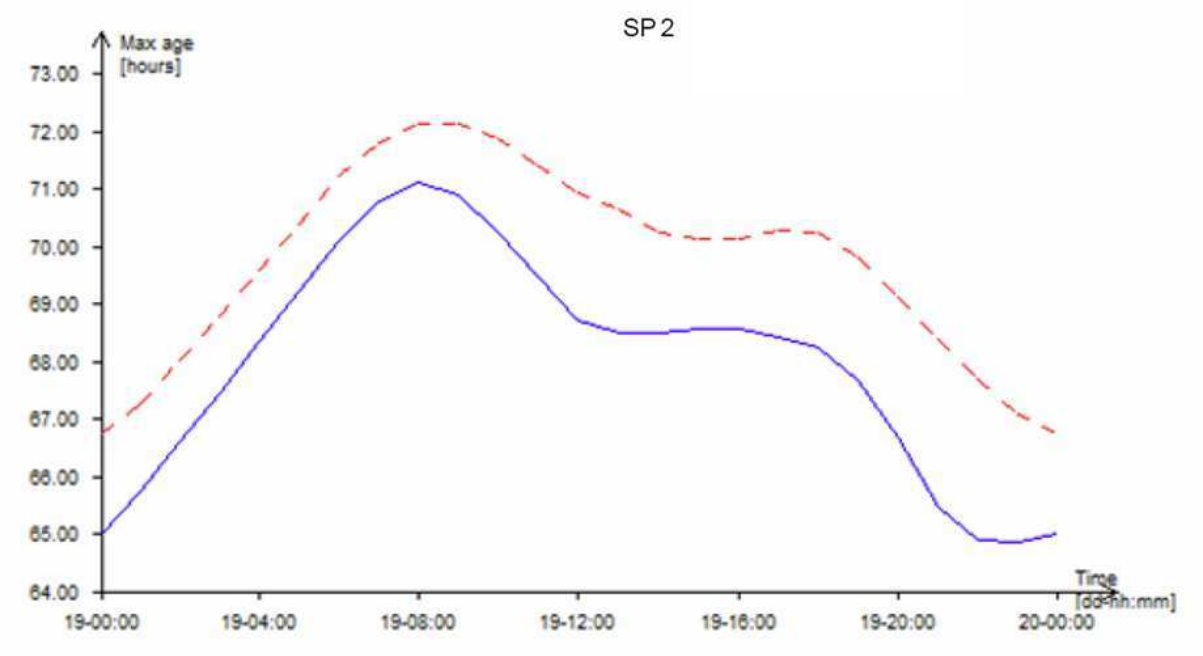
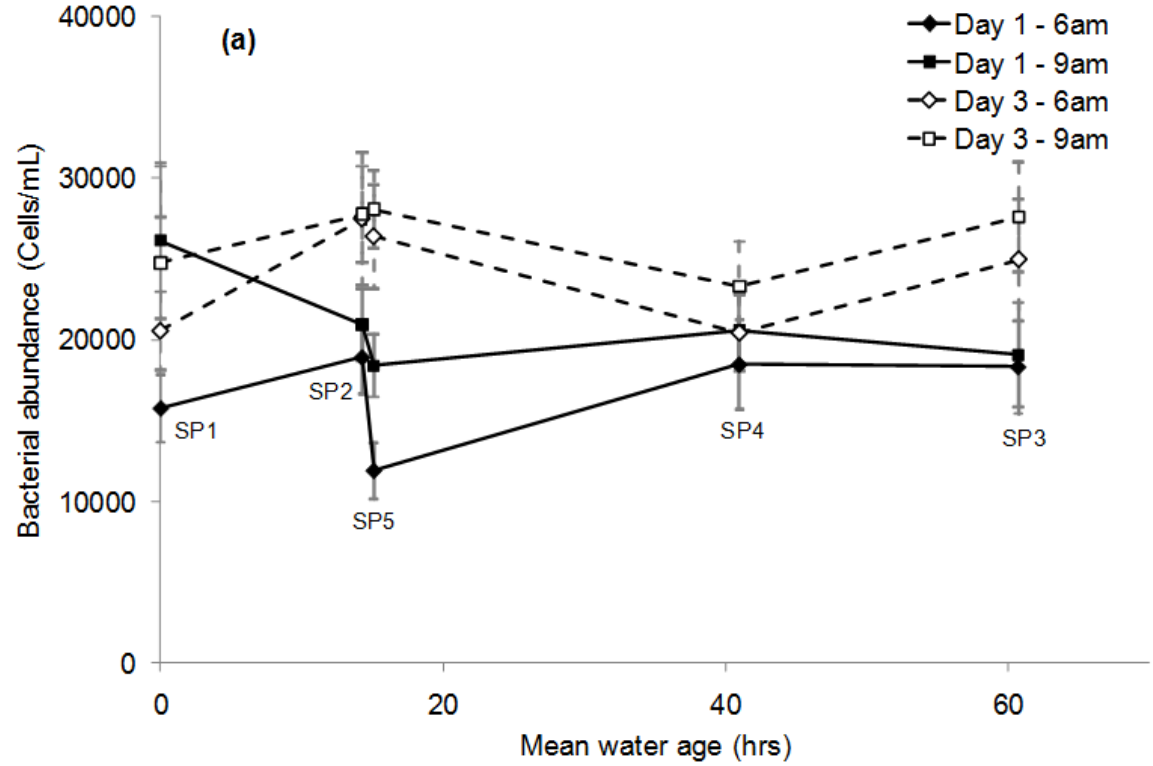


Fig. 3 - 24 hour profile of maximum water age at the start (node 1) and end (node2) of the pipe length immediately upstream of sampling point 2.



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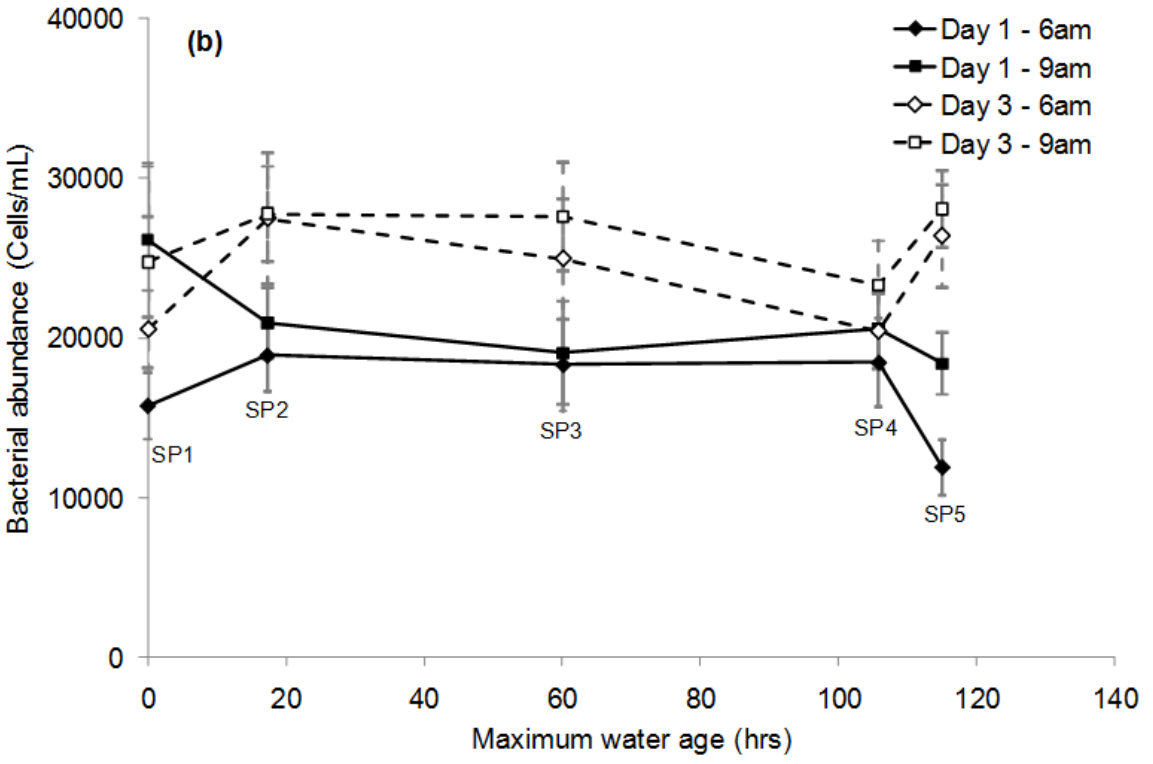
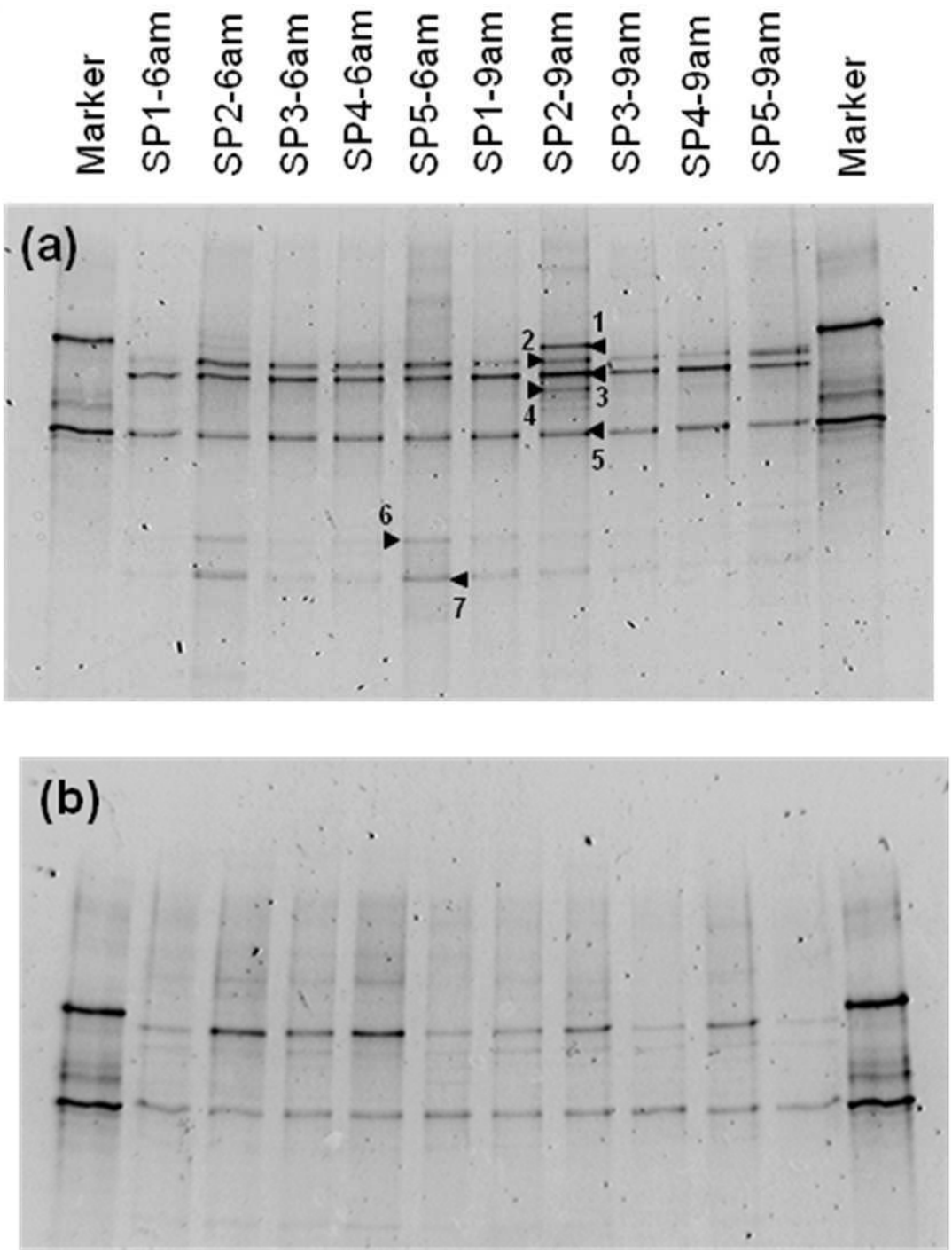


827

828 **Fig. 4** – Variation in total mobile / suspended bacterial cell counts between days (1 and 3)
829 and over time (6am and 9am) of sampling in relation to a) mean- and b) maximum-water age.
830 Error bars indicate +/- 1 standard deviation (n=3, at each site, day and time point).

831



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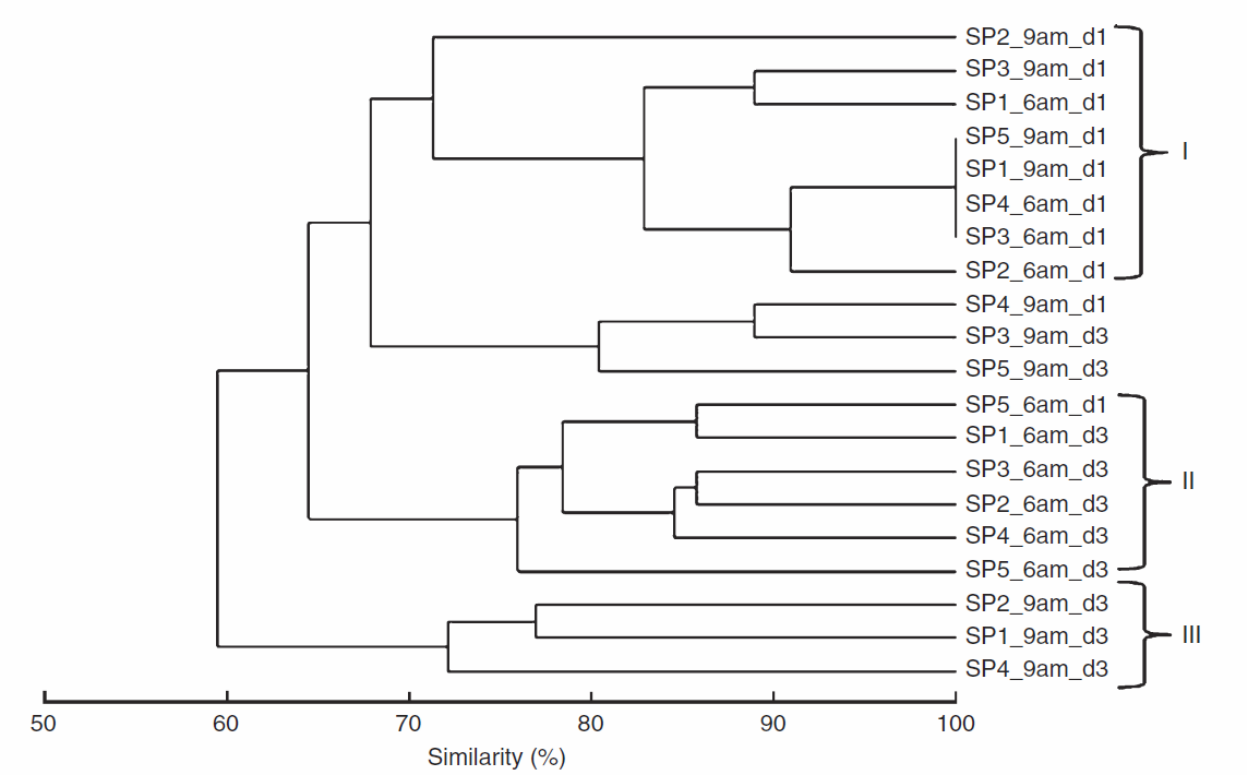
833

834 **Fig. 5** - DGGE analysis of PCR amplified 16S rRNA gene fragments derived from drinking
835 water samples collected at five sampling locations (SP1 to SP5) on two days a) Monday (day
836 1) and b) Wednesday (day 3) at two time points (6 and 9 am). Lanes are as indicated. The
837 bands 1 to 7 corresponds to *Acinetobacter* sp., *Nevskia* sp., *Pseudomonas* sp., *Pseudomonas*
838 sp. *Escherichia* sp., *Bosea* sp. and *Bosea* sp., respectively.

839



840

841 **Fig. 6** - Cluster analysis of DGGE profiles of 16S rRNA gene fragments obtained from five
842 sampling locations (SP1 to SP5), sampled on days 1 and 3 at 6 and 9 am. Samples are as
843 indicated.

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Tables

Table 1 - Calculated mean and maximum water age, relative to SP1. The mean and maximum water age values were calculated based on the model developed by Machell *et al*. (2009); see text for details.

Sampling Point	Mean Water Age (hours)	Maximum Water Age (hours)	Mix
SP1	0	0	Minimal
SP2	14	17	Minimal
SP3	60	60	Minimal
SP4	41	106	81% 16 to 40 hours old; 19% 58 to 112 hours old
SP5	15	115	96% 0 to 16 hours old; 4% 112 to 136 hours old

Table 2 - Range of standard physico-chemical and microbiological parameters for water samples collected from the five locations within the water distribution network.

Parameter	**Range** (min – max)
Temperature (^{o}C)	15.2 - 18.4
pH (-)	7.1 - 7.3
Conductivity (µS cm^{-1})	189 - 219
Turbidity (NTU)	0.1 - 1.61
True colour (mg l^{-1} Pt/Co)	0.9 - 2.5
Chlorine free (mg l^{-1} Cl)	0.04 - 0.05
Chlorine total (mg l^{-1} Cl)	0.04 - 0.15
Iron (µg l^{-1} Fe)	15 - 638
Aluminium (µg l^{-1} Al)	28 - 91
Manganese (µg l^{-1} Mn)	2.0 - 4.6
Total coliforms (CFU 100ml^{-1})	0
Escherichia coli (CFU 100ml^{-1})	0
Fecal *Steptococcus* (CFU 100ml^{-1})	0
Clostridium perfringens (CFU 100ml^{-1})	0
Viable bacterial count – 2 days at 37 ^{o}C (CFU ml^{-1})	0 - 34
Viable bacterial count – 3 days at 22 ^{o}C (CFU ml^{-1})	0 - 42

Table 3 - 16S rRNA gene sequences retrieved from DGGE bands and their closest phylogenetic affiliation (SP1 to SP5 are sampling locations, d1 and d3 are day1 and 3, respectively).

Sampling locations	Closest relative in GenBank (Accession Number)	% Identity	Found Elsewhere in Drinking Water / DW Biofilms[a]	References
SP2 (d1, 6 am & 9 am)	*Acinetobacter venetianus* (FJ608115); *Acinetobacter* sp. (FJ876296)	99	*A. calcoaceticus* in drinking water (Portugal) *Acinetobacter* sp. in WDS (Greece)	Simoes *et al.* (2007) Kormas *et al*. (2009)
SP1, SP2, SP3, SP4, SP5 (d1 & d3, 6 am & 9 am)	*Nevskia ramosa* (AJ001343) *Nevskia* sp. (AB426557)	91 - 97	*N. ramosa* in chlorinated drinking water (USA) *N. ramosa* in model WDS (USA) N. ramosa in drinking water (USA)	Williams *et al*. (2004) Keinanen-Toivola *et al*. (2006) Revetta *et al*. (2009)
SP1, SP2, SP3, SP4, SP5 (d1 & d3 6 am & 9 am)	*Pseudomonas* sp. (GQ417894)	99 - 100	*P. aeruginosa* isolated from drinking water (Brasil) *Pseudomonas* sp. in chlorinated drinking water (USA) *Pseudomonas* sp. in drinking water biofilms (South Africa)	Silva *et al*. (2008) Williams *et al*. (2004) September *et al*. (2007)
SP1, SP5 (d1, 6 am) SP2, SP4 (d1, 9 am) SP1, SP3, SP5 (d3, 6 am) SP3 (d3, 9 am)	*Pseudomonas* sp. (DQ316117)	90	*Pseudomonas* sp. in chlorinated drinking water (USA) *Pseudomonas* sp. in drinking water biofilms (South Africa)	Williams *et al*. (2004) September *et al*. (2007)
SP1, SP2, SP3, SP4, SP5 (d1, 6 am & 9 am) (d3, 6 am & 9 am)	*Escherichia* sp. (GQ423062)	99 - 100	*E. coli* in private water supplies (UK) *E. coli* outbreak in drinking water supplies (UK) *E. coli* in several water supplies (France, UK, Portugal and Lativa)	Richardson *et al*. (2009) Nichols *et al*. (2009) Juhna *et al*. (2007)
SP2, SP3, SP5 (d1, 6 am) SP1, SP2, SP5 (d1, 9 am) SP1, SP2, SP3, SP4, SP5 (d3, 6 am) SP1 (d3, 9 am)	*Bosea* sp. (AB480419)	93 - 98	*Bosea* sp. found in drinking water (Switzerland) *Bosea* sp. found in treated water	Thomas *et al*. (2007) Rapala *et al*. (2006)
SP1, SP2, SP3, SP4, SP5 (d1, 6 am) SP1, SP2, SP3, SP5 (d1, 9 am)	*Bosea* sp. (AB480419)	100	*Bosea* sp. found in drinking water (Switzerland) *Bosea* sp. found in treated water	Thomas *et al*. (2007) Rapala *et al*. (2006)

[a] Organisms related to those identified in this study have previously also been found in other drinking water distribution systems, as indicated.