Detection, integration and persistence of aeromonads in water distribution pipe biofilms

A.-M. Bomo, M. V. Storey and N. J. Ashbolt

ABSTRACT

The occurrence of *Aeromonas* spp. within biofilms formed on stainless steel (SS), unplasticized polyvinyl chloride (uPVC) and glass (GL) substrata was investigated in modified Robbins Devices (MRD) in potable (MRD-p) and recycled (MRD-r) water systems, a Biofilm Reactor™ (BR) and a laboratory-scale pipe loop (PL) receiving simulated recycled wastewater. No aeromonads were isolated from the MRD-p whereas 3–10% of SS and uPVC coupons (mean 3.85 CFU cm^{-2} and 12.8 CFU cm^{-2}, respectively) were aeromonad-positive in the MRD-r. Aeromonads were isolated from six SS coupons (67%) (mean 63.4 CFU cm^{-2}) and nine uPVC coupons (100%) (mean 6.50×10^2 CFU cm^{-2}) in the BR™ fed with recycled water and from all coupons (100%) in the simulated recycled water system (PL). Mean numbers of aeromonads on GL and SS coupons were 5.83×10^2 CFU cm^{-2} and 8.73×10^2 CFU cm^{-2}, respectively. No isolate was of known human health significance (i.e. *Aeromonas caviae*, *A. hydrophila* or *A. veronii*), though they were confirmed as *Aeromonas* spp. by PCR and fluorescence *in situ* hybridization (FISH). Challenging the PL biofilms with a slug dose of *A. hydrophila* (ATCC 14715) showed that biofilm in the PL accumulated in the order of 10^3–10^4 *A. hydrophila* cm^{-2}, the number of which decreased over time, though could not be explained in terms of conventional 1st order decay kinetics. A sub-population of FISH-positive *A. hydrophila* became established within the biofilm, thereby demonstrating their ability to incorporate and persist in biofilms formed within distribution pipe systems. A similar observation was not made for culturable aeromonads, though the exact human health significance of this remains unknown. These findings, however, further question the adequacy of culture-based techniques and their often anomalous discrepancy with direct techniques for the enumeration of bacterial pathogens in environmental samples.

Key words | *Aeromonas* spp., biofilm, drinking water, pathogen accumulation, recycled water, water distribution pipes

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INTRODUCTION

Bacteria belonging to the genus *Aeromonas* have long been known to cause disease in fish, reptiles and amphibians (Hazen *et al.* 1978). In more recent years, however, their role as opportunistic pathogens implicated in human illnesses such as gastroenteritis, wound infections, septicaemia and soft tissue conditions, has gained increasing interest (Deodhar *et al.* 1991; Janda *et al.* 1994; Baloda *et al.* 1995; Percival & Walker 1999; Essers *et al.* 2000). Since 2003, various public water utilities in the United States have started a screening survey for the presence of *Aeromonas* in their distribution pipe water (EPA 2003). The motile mesophilic aeromonads implicated in human illnesses include *A. caviae*, *A. veronii* (biotype *sobria*) and *A. hydrophila* (Deodhar *et al.* 1991; Janda & Abbot 1998). Strains of *A. hydrophila* are capable of causing infections in both fish and humans, whilst the non-motile *A. salmonicida* is known solely as a fish pathogen (Lunder & Håstein 1990).

Aeromonads occur widely in nature and have been isolated from soils, foods and many types of water, both fresh and marine (Hazen *et al.* 1978; Nakano *et al.* 1990; Ashbolt *et al*. 1995). Aeromonads are also found in high numbers in raw sewage and sewage effluents, and are capable of growing in natural waters in the presence of sufficient nutrients and temperatures (Rippey & Cabelli 1985; Poffe & Op de Beeck 1991). It has been proposed therefore that the presence of elevated numbers of aeromonads may be indicative of faecal pollution and nutrient-rich (eutrophic) conditions (Araujo *et al*. 1991; Poffe & Op de Beeck 1991). Furthermore, a positive correlation has been reported between freshwater eutrophication and the presence of potentially virulent aeromonads, mainly *A. hydrophila* and *A. veronii* (Ashbolt *et al*. 1995). Araujo *et al.* (1991) have observed that *A. caviae* predominate in sewage and in waters with a high degree of faecal pollution, whereas, in less polluted waters (fresh or marine), *A. caviae* and *A. hydrophila* were equally distributed, with *A. sobria* the dominant species in non-polluted waters. Potentially virulent aeromonads have also been isolated from drinking water supplies (Kühn *et al*. 1997*a*), which may represent strains that cause wound infection (Gold & Salit 1993), but appear unlikely to be important gastrointestinal strains (Havelaar *et al*. 1992; WHO 2002; Borchardt 2003). Nonetheless, aeromonads have been isolated from biofilms in water distribution systems (Holmes & Nicolls 1995; Walker *et al*. 1995; Chauret *et al*. 2001), increasing their persistence over many years (Kühn *et al*. 1997*b*) and making them a potential health risk for consumers of distribution pipe water, particularly the immunocompromised.

Biofilms provide an advantageous environment for bacteria in otherwise oligotrophic environments, offering them an increased flux of nutrients that increases both their chances of survival and regrowth potential. Biofilms that form on pipe surfaces within a distribution system may therefore act as a reservoir for microbial pathogens to persist for prolonged periods of time and even recolonize the bulk water environment (Szewzyk *et al.* 2000). Emerging waterborne pathogens such as *Helicobacter pylori* (Mackay *et al.* 1998), enterohaemorrhagic *E. coli* (EHEC) (Szewzyk *et al.* 1994), *Salmonella typhimurium* (Armon *et al.* 1997) and *Campylobacter* spp. (Buswell *et al.* 1998) have been shown to persist within biofilms formed in experimental laboratory systems and may therefore have the potential to accumulate and persist within pipe biofilms formed within a distribution system. Though culturable forms of these pathogens have not been isolated from municipal water distribution systems, the opportunistic pathogens aeromonads have (van der Kooij & Hijnen 1988; Gavriel *et al*. 1998; Szewzyk *et al*. 2000). Biofilms can therefore act as a reservoir for aeromonad growth, providing an environment in which they can accumulate and persist. Problems may then arise in distribution systems through detachment of biofilm from the pipe surface, either by continous erosion of small portions of biofilm or more rapid and massive loss of biofilm by sloughing (Characklis 1990). Such biofilm detachment could contribute to bacterial contamination of the bulk water and thereby present a potential health risk to consumers. Thus, knowledge of biofilm growth and pathogen accumulation in distribution pipe systems should be an important objective for good water distribution management strategies.

Despite the ubiquitous isolation of *Aeromonas* from the bulk water phase of distribution systems worldwide, a limited amount of work has focused on the direct examination of aeromonads in biofilms formed within distribution pipe systems (Holmes & Nicolls 1995; Walker *et al*. 1995; Chauret *et al*. 2001). The purpose of this study was therefore to investigate various methods that can be used to screen for aeromonads in biofilms from different water sources and to investigate the integration and persistence of *A. hydrophila* biofilms in a laboratory-scale simulated non-potable water distribution system.

MATERIALS AND METHODS

Potable water biofilm study

Field studies were carried out at a biofilm sampling site (BSS) located within the Rouse Hill Development Area (RHDA), New South Wales (Storey & Ashbolt 2001), the site of Australia's first full-scale dual reticulation (potable

and recycled water) system. Biofilms were collected from two different types of devices within the BSS; modified Robbins Devices (MRDs) and a Biofilm Reactor™ (BR™) (AWT, Environment, Science and Technology, Sydney, Australia) described in greater detail elsewhere (Storey & Ashbolt 2002). The MRDs were 150 mm in diameter and contained removeable unplasticized polyvinyl chloride (uPVC) coupons and grade 304 bright annealed stainless steel (SS) coupons placed in a helical pattern over the length of the device (1.2 m). Care was taken not to compromise the hydraulic integrity of the device through the overlapping of coupons.

MRDs were connected in-line within cement-lined potable (MRD-p) and recycled (MRD-r) water distribution pipes, whilst the BR™, also containing SS and uPVC coupons, was connected to the recycled water distribution pipe. The BR™ was a modified Couette device, or 'annular reactor', which was configured to produce similar hydraulic conditions to those that would be encountered within the distribution system (Reynold's number, $Re \sim 10^3$). The concentration of free and total chlorine was measured in all devices at 10 min intervals by Dulcotest® CGE 2 mg l^{-1} sensors (ProMinent® Dosiertechnik, GmbH, Heidelberg, Germany) fitted in an in-line sensor housing (ProMinent® GmbH), while pH was measured by Dulcotest® sensors (ProMinent® GmbH). The hydraulic demand was measured by MAGFLO® electromagnetic flow meters Type MAG 3100 fitted with a MAG 3100 signal converter and SENSORPROM™ memory unit (Danfoss Instrumentation Aust. Pty. Ltd), while water temperature was measured by Type A stainless steel thermocouples (Pyrosales Pty. Ltd, Sydney).

Biofilms were allowed to accumulate on coupon surfaces in each device for a period of 10 weeks. After this time, coupons were removed from MRDs and the BR™ and placed in sterile stomacher bags containing 30 ml of $\frac{1}{4}$ strength Ringers solution. Biofilms were then removed from coupon surfaces using a combination of sonication and stomaching as described elsewhere (Storey & Ashbolt 2002). Both techniques resulted in a greater than 3-log removal of biofilm bacteria from coupon surfaces, whilst at the same time, not having any measurable effect on the viability of biofilm bacteria (Storey 2002).

Recycled water biofilm study

Both the MRD-p and MRD-r within the BSS were serviced with potable water only throughout the duration of this study. In order to study biofilms formed in recycled wastewater, a laboratory-scale pipe loop (PL) system receiving simulated recycled wastewater was constructed (Storey & Ashbolt 2002). The PL consisted of 100 mm uPVC piping designed in a U-configuration that permitted the re-circulation of water by a peristaltic pump. The device, which resembled a MRD, had a total volume of 60 litres, and contained SS and and glass (GL) coupons that could be removed from the PL throughout the duration of the experiment. For each experimental run, the PL was inoculated with diluted primary effluent (10% in potable water) (total organic carbon, TOC: 68 mg l^{-1}, total nitrogen, TN: 40.3 mg l^{-1}; total phosphate, TP: 24.8 mg l^{-1}). Diluted effluent was then re-circulated throughout the system at a linear velocity equivalent to that within the field (1 cm sec^{-1}) for a period of 21 days to precondition coupon surfaces with water containing equivalent nutrient levels to those expected in recycled wastewater. For the remainder of each experimental period, the PL was connected directly to tap water (temperature: 22–25°C) supplemented with sterile (autoclaved) primary effluent (0.05–0.1%) and operated in a single pass flow through the system. After 8 weeks of biofilm accumulation, coupons were removed from the PL and the biofilm removed from coupons using methods described previously.

Biofilm characterization and enumeration and identification of aeromonads

Biofilm homogenates were characterized by total protein (NanoOrange® Protein Quantitation Kit) and carbohydrate (Phenol-sulfuric) content, heterotrophic plate counts (HPC on R2A agar) and total bacteria (LIVE/DEAD *Bac*Light™ Bacterial Viability Kit) as described elsewhere (Storey & Ashbolt 2002). Results were expressed per cm^2 of biofilm coverage on coupon surfaces. The numbers of aeromonads in biofilm homogenates were determined using methods described in Ashbolt *et al.* (1995). Appropriate volumes of biofilm homogenate were membrane-filtered (0.45 μm nitrocellulose filter,

Millipore) and filters placed on m*Aeromonas* Agar Base (CM833, Oxoid Ltd, UK) supplemented with 5 mg l^{-1} ampicillin. The plates were incubated at 37°C and colonies enumerated after 24 h. Presumptive aeromonad colonies were picked from the primary isolation plates, re-streaked on to Tryptone Soya Agar (TSA) (Oxoid Pty. Ltd, UK) and incubated for 48 h at 37°C. Oxidase-positive isolates were then chosen for further identification using biochemical testing, PCR and fluorescence *in situ* hybridization (FISH).

Presumptive aeromonad isolates were identified using the eight biochemical tests (esculin hydrolysis, Voges-Proskauer, H_2S production, arabinose fermentation, salicin fermentation, gluconate oxidation and production of gas from glycerol and glucose) described by Popoff & Veron (1976), Poffe & Op de Beeck (1991) and Ashbolt *et al*. (1995).

For PCR analysis, isolates were grown overnight in nutrient broth and DNA extracted from 1 ml of the cell suspension using QIAamp® DNA Mini Kit (Qiagen Pty Ltd, Victoria, Australia). PCR was run using species-specific forward primers for *A. hydrophila* (5′ GAA AGG TTG ATG CCT AAT ACG TA 3′) and *A. veronii* (5′ GAG GAA AGG TTG GTA GCT AAT AA 3′) (Dorsch *et al*. 1994). A general forward *Aeromonas* genus primer (5′ GAG TTT GAT CCT GGC TCA G 3′) developed by Dorsch & Stackebrandt (1992) was also used for identification of isolates at the genus level. A reverse *Aeromonas* genus primer (5′ CGT GCT GGC AAC AAA GGA CAG 3′) (Dorsch *et al*. 1994) was used in combination with all three forward primers. All PCRs were run under the following conditions: 94°C for 2 min, 92°C for 15 sec, cycled 30 times at 45°C for 15 sec, then at 72°C for 20 sec, 45°C for 45 sec, 72°C for 5 min and 25°C for 1 min. The reaction mixtures in all tubes (40 μl capillary tubes) contained 2 μl of DNA, 20 *η*g primer and 0.25 mmol l^{-1} dNTP, 4 μl of 10 × reaction buffer and 2.5 mmol l^{-1} of $MgCl_2$. Distilled water was added to the tubes to reach a final volume of 40 μl. One unit of Taq polymerase (Red Hot® DNA Polymerase, ABgene, Surrey, UK) was added last to each tube. PCR products were analysed by agarose gel electrophoresis (1%) that included a molecular weight standard (100 bp DNA ladder, N3231S, Biolabs Inc., New England). All primers were purchased from Genset Pacific Pty. Ltd, Lismore, Australia.

Confirmation of presumptive aeromonad isolates to genus level was performed by FISH using a CY3-labelled rRNA-targeted oligonucleotide probe (5′-CTA CTT TCC CGC TGC CGC C-3′) complementary to a region of the 16S rRNA of all sequenced aeromonads except for *A. schubertii* (Kampfer *et al*. 1996) (Genset SA, Paris, France). Cells were fixed in 3% paraformaldehyde at 4°C for 1–3 h and washed in PBS. Ten microlitres of the fixed cell suspension were added to wells on microscope slides that had been pretreated with chlorinated organopolysiloxane in heptane (Sigmacote SL2) (Sigma Chemical CO, St Louis, USA). The cell suspension was air-dried and the cells hybridized using the conditions as described by Manz *et al.* (1992) and Kampfer *et al*. (1996). Samples of 10 μl hybridization solution (35% formamide, 0.9 mol l^{-1} NaCl, 20 mmol l^{-1} Tris pH 7.2, 0.01% SDS, 5 *η*g probe) were applied to each well on the slide and incubated for 1.5 h at 46°C in a humidified chamber. The probe was removed from the slide by adding 2 ml of prewarmed washing buffer (20 mmol l^{-1} Tris, 0.01% SDS, 40 mmol l^{-1} NaCl, 5 mmol l^{-1} EDTA) to the slide and immersing the slide in washing buffer for 20 min at 46°C. Slides were counterstained with DAPI (1 μg ml^{-1}) and mounted in Citiflour AF2 (Citiflour Ltd, London, UK). Homogenates from the BSS were also pooled together and mixed well for FISH analyses as described above. All slides were examined by epifluorescence microscopy (EFM) (Leitz Laborlux S™, Leitz Corp., Germany).

Integration and persistence of *A. hydrophila* within biofilms

Biofilms were allowed to accumulate on the coupons in the PL for 8 weeks using experimental conditions described previously. The loop was then slug-dosed with a suspension (1×10^8 CFU ml^{-1}) of *A. hydrophila* (ATCC 14751). The bacterial cells were allowed to accumulate in the biofilm by re-circulating the cell suspension in the loop for a period of 24 h, after which time the loop was operated in a single pass mode. On days −1, 0, 1, 2, 3, 6, 10, 20 and 30, GL and SS coupons were removed from the system. Duplicate coupons of each material were

removed on each sampling day, one for analyses of biofilm homogenate using the same parameters and methods as described earlier, the second for FISH. Hybridization conditions were the same as described previously. Each hybridization was performed with the CY3-labelled *Aeromonas* probe and a general CY3 labelled bacterial probe (EUB 338) (Giovannoni *et al.* 1988). In addition, negative controls were undertaken to confirm probe specificity and measure amounts of background fluorescence. Coupons were counterstained with DAPI (1 μg ml^{-1}), mounted in Citiflour AF2 (Citiflour Ltd, London, UK) and examined by EFM. For statistical evaluation 40–60 fields of view per probe and coupon were enumerated, with results expressed as cells cm^{-2}.

Water samples were also taken from the pipe loop on the same days as biofilm was collected from the system and analysed for the occurrence of culturable aeromonads using methods described previously.

Statistical analyses

All bacterial counts were log10 transformed prior to their analysis. In an attempt to account for the inherently heterogeneous biofilm coverage and composition on coupon surfaces (Storey & Ashbolt 2002), aeromonad numbers from the BR™ device were normalized against total biofilm protein and total carbohydrate content. Comparisons of results were carried out using analysis of variance (ANOVA) with Tukey Kramer HSD test for multiple comparisons with statistically significant differences for $P<0.05$. Linear regression was carried out on the data from the integration and persistence study to determine whether first order kinetics (Equation 1) could be used to describe the declining aeromonad numbers observed within coupon biofilms.

$$dC/dt = -kC$$

$$C(t) = (C_n)e^{-kt} \quad (1)$$

All statistical analyses were undertaken using JMP v. 3.2.2 (SAS Institute, Cary, NC, USA).

RESULTS

General water quality in different systems

Total and free chlorine were measured in the MRD-p at concentrations of 0.24 ± 0.14 mg l^{-1} and 0.04 ± 0.14 mg l^{-1}, respectively. Within the MRD-r, total and free chlorine were measured at 0.19 ± 0.12 mg l^{-1} and 0.03 ± 0.03 mg l^{-1}, respectively. Water pH was recorded at 7.9 ± 0.26 in the MRD-p and 7.8 ± 0.42 in the MRD-r. Water temperature was 21.5°C and 22.4°C in MRD-p and MRD-r, respectively. The hydraulic demand in the MRD-p was 1.1 ± 0.92 l sec^{-1} and 0.46 ± 0.53 l sec^{-1} in the MRD-r, equating to linear velocities of 6.2 ± 5.2 cm sec^{-1} and 2.6 ± 3.0 cm sec^{-1}, respectively.

Quantification of aeromonads in different systems

No aeromonads were detected in biofilms formed in the MRD-p, whereas from a total of 30 coupons investigated for the two substrata in the MRD-r, aeromonads were isolated from one SS coupon (3.3%) and three uPVC coupons (10%) (detection limit of 25 CFU cm^{-2}) (Table 1). On the *Aeromonas*-positive coupons, the numbers of aeromonads were still relatively high when compared with the total viable count of bacteria (0.02%). Aeromonads were isolated from biofilms formed on six SS coupons (67%) and nine uPVC coupons (100%) in the BR™ (Table 1). Due to the very low numbers of aeromonads observed in both MRDs, comparisons of differences between substrata (SS and uPVC) were only undertaken for the BR™. Here a significantly higher ($P = 0.03$) number of aeromonads were culturable from uPVC compared with SS coupons. Aeromonads were recovered from biofilms formed on all coupons in the PL (Table 1). A comparison of pooled biofilm homogenates from four SS and four GL coupons in the PL showed no significant ($P>0.05$) difference in the number of aeromonads between substrata. Neglecting different substrata and combining all the aeromonad results due to water quality (potable water vs. recycled wastewater) showed that there was significantly more aeromonad growth in biofilms formed within the PL compared with the BR™ ($P = 0.005$).

Table 1 | Average numbers of *Aeromonas* in biofilm and characterization of biofilm homogenates (HPC, total bacterial counts, total protein and total carbohydrate) from different water systems and substrata

Water source	Sampling device	Material	Number of coupons investigated	Aeromonads (CFU cm^{-2} ± 1SD)	Heterotrophic plate counts (HPC) (CFU cm^{-2} ± 1SD)	Total bacterial counts (cells cm^{-2} ± 1SD)	Total protein concentration ($\mu g\ cm^{-2}$ ± 1SD)	Total carbohydrate concentration ($\mu g\ cm^{-2}$ ± 1SD)
Potable water	MRD-p	SS	6	ND	5.18×10^2 ($\pm 9.25 \times 10^2$)	2.18×10^6 ($\pm 6.11 \times 10^5$)	4.27 ± 3.31	4.51 ± 2.25
	MRD-p	uPVC	6	ND	2.24×10^3 ($\pm 6.89 \times 10^3$)	2.03×10^6 ($\pm 3.49 \times 10^5$)	5.53 ± 3.26	4.75 ± 3.10
	MRD-r	SS	30	3.85×10^0 ($\pm 2.11 \times 10^1$)	1.19×10^3 ($\pm 1.72 \times 10^3$)	1.56×10^6 ($\pm 1.01 \times 10^6$)	5.33 ± 2.56	7.26 ± 3.23
	MRD-r	uPVC	30	1.28×10^1 ($\pm 5.98 \times 10^1$)	6.21×10^3 ($\pm 8.17 \times 10^3$)	2.09×10^6 ($\pm 2.42 \times 10^6$)	6.17 ± 2.80	7.18 ± 3.27
	BR-r	SS	9	6.34×10^1 ($\pm 1.53 \times 10^2$)	3.95×10^3 ($\pm 4.05 \times 10^3$)	1.17×10^6 ($\pm 2.60 \times 10^5$)	12.87 ± 4.22	11.85 ± 4.49
	BR-r	uPVC	9	6.50×10^2 ($\pm 1.26 \times 10^3$)	1.11×10^4 ($\pm 1.17 \times 10^4$)	4.82×10^5 ($\pm 9.02 \times 10^4$)	12.71 ± 4.74	12.29 ± 5.11
Recycled water	PL	GL	18	3.04×10^2 ($\pm 1.01 \times 10^3$)	4.71×10^5 ($\pm 1.52 \times 10^5$)	9.66×10^6 ($\pm 3.25 \times 10^6$)	68.99 ± 23.19	33.69 ± 8.22
	PL	SS	18	8.73×10^2* ($\pm 6.9 \times 10^1$)	3.74×10^5 ($\pm 2.80 \times 10^5$)	7.94×10^6 ($\pm 3.12 \times 10^6$)	138.34 ± 26.43	61.82 ± 16.38
	PL	GL		5.83×10^2* ($\pm 2.4 \times 10^2$)	Not determined	Not determined	Not determined	Not determined

ND, Not detected or numbers below detection limit of 25 CFU cm^{-2}.
*Pooled biofilm homogenate from four coupons. Comparison of aeromonad numbers on SS and GL in PL were based only on pooled biofilm homogenates.

Characterization of biofilm from different systems

There were significantly more total ($P<0.05$) and culturable bacteria ($P<0.0001$) recovered from biofilms formed in the BR™ than from the two MRDs (Table 1), though no such difference was observed between MRDs. These findings were also supported by a general trend of higher total biofilm protein and carbohydrate content in the BR™, although these differences could not be supported statistically ($P>0.05$). A comparison of substrata showed that significanly more ($P=0.02$) culturable bacteria were present in biofilm from uPVC than from SS coupons, although this observation could not be supported by the total number of bacterial cells or total biofilm protein and carbohydrate content. Furthermore, there was significantly more ($P<0.0001$) biofilm biomass recovered from the PL than any other device and significantly ($P=0.02$) more total and culturable bacteria on GL than SS coupons. In contrast, the opposite was observed for total biofilm protein and carbohydrate content (Table 1).

Normalizing aeromonad counts from each coupon in the BR™ device against the corresponding total protein and total carbohydrate concentrations from each coupon (CFU aeromonads μg per protein cm^{-2} and CFU aeromonads μg per carbohydrate cm^{-2}, respectively) did not show any significant differences between substrata (SS and uPVC).

Identification of aeromonads

Biofilm homogenates from potable water systems

Only half of a total of 40 presumptive aeromonad colonies isolated from potable water systems grew on TSA agar, even after an extended incubation time (>48 h). Of the isolates, 13 were oxidase-positive and these isolates were therefore used for further identification by biochemical testing, FISH and PCR. Of the eight biochemical tests undertaken, only the test for H_2S gas production provided a positive result (61.5% positive). Three positive controls (*A. hydrophila* ATCC 14715, *A. caviae* ATCC 15468 and *A. veronii-sobria* ATCC 9071) were used and each performed as expected for each test, yet isolate speciation based on eight phenotypic characteristics was inconclusive. Seven of 13 (54%) of the atypical phenotype isolates were confirmed as aeromonads by FISH and 7 of 13 (54%) by PCR, with a 70% agreement between both techniques. Further testing with species-specific primers confirmed that the *Aeromonas* isolates were not of human health significance (i.e. not *A. hydrophila*, *A. veronii-sobria* or *A. veronii-veronii*).

Twenty sub-samples of pooled biofilm homogenates were also screened for aeromonads using FISH. Within each sub-sample, aeromonads were detected in 49% of biofilms sourced from the MRD-r, and 35% from biofilm homogenates sourced from the MRD-p.

Biofilm homogenates from recycled wastewater

Only PCR and FISH were used to identify aeromonads cultured from the recycled water system. Twenty-one presumptive *Aeromonas* colonies were picked and re-streaked on to TSA agar. All isolates showed good growth on the TSA plates and were used in further identification. Nine of 21 (43%) of the isolates were confirmed as aeromonads by PCR and 10 of 21 (48%) by FISH, with a 76% agreement between both techniques.

Integration and persistence of *A. hydrophila* in biofilms

Background levels of aeromonads were detected in biofilms prior to seeding the simulated recycled water distribution system (PL) with *A. hydrophila*. FISH-positive aeromonads were detected in the order of 10^3–10^4 cm^{-2} on SS and GL substrata, respectively, at day −1, whilst culturable aeromonads were in the order of 10^2 cm^{-2} on both GL and SS substrata. After seeding, biofilms accumulated between 10^3 and 10^4 culturable and FISH-positive *A. hydrophila* cm^{-2} (Figure 1a and b). CFU numbers for culturable aeromonads initially declined rapidly, then more gradually (days 3–30). At the end of the experimental period, there was no statistically significant ($P>0.05$) difference in culturable aeromonad numbers recovered from biofilms on day -1 and day 30 on both GL and SS substrata. No significant differences ($P>0.05$) in biofilm

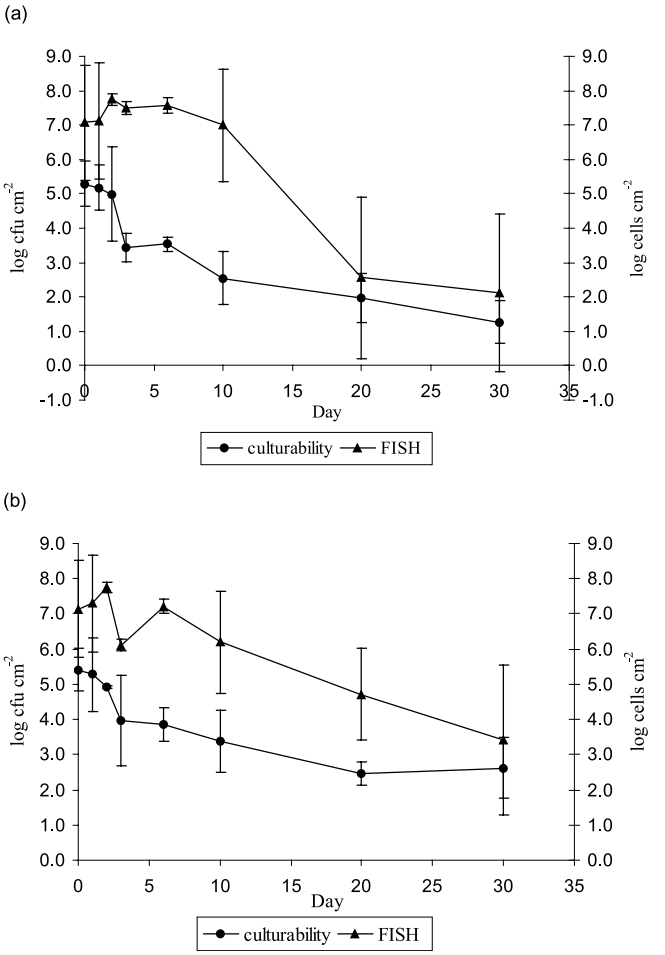

Figure 1 | Log-transformed numbers of aeromonads in biofilm formed on (a) GL coupons and (b) SS coupons. *Aeromonas* numbers determined by culturability (CFU cm^{-2}) ($n=2$) and by FISH (cells cm^{-2}) (Values based on the mean of cells counted in 60 separate areas on each coupon.) Error bars represent one standard deviation.

biomass (HPC, total bacterial counts, total protein and total carbohydrate) were observed between sampling days or between substrata (SS and GL) (data not shown).

Throughout the experiment, aeromonads were determined in higher numbers by FISH than by standard culture techniques (Figure 1a and b). FISH-positive cells remained at high levels until day 10, followed by a more gradual decline. On day 30, aeromonads appeared to establish themselves within biofilms formed on SS coupons; the number of FISH-positive aeromonads was significantly higher ($P<0.0001$) than that found prior to seeding the system (day -1). No such observation, however, was made for GL coupons.

A line of regression (Figures 2 and 3) was fitted to the persistence data to ascertain whether the reduction of *A. hydrophila* during the experimental period could be described in terms of 1st order kinetics. With the FISH method, the log-linear correlation factor (r^2) was 0.57 for GL (Figure 2a) and 0.50 for SS coupons (Figure 2b), whilst for the culture-based method, r^2 was 0.70 for GL (Figure 3a) and 0.57 for SS coupons (Figure 3b). The gradual decline observed between days 10 and 20 by FISH (Figure 1a and b) contributed most to the log-linearity with a correlation factor of 0.55 for GL and 0.23 for SS coupons. For the remainder of the period (days 0–10 and days 20–30) numbers of aeromonads persisted at relatively stable levels. The same pattern was observed with the culture-based data, with the highest log-linear correlation for the time period with a gradual decline (days 3–30 for GL, days 1–20 for SS) (Figure 1a and 1b).

Before seeding the PL, no aeromonads were detected in the bulk water (<1 CFU 100 ml^{-1}). After seeding, numbers of aeromonads increased to 1×10^8 CFU ml^{-1} in the bulk water, although they were not detected after day 6 (detection limit 1 CFU 100 ml^{-1}).

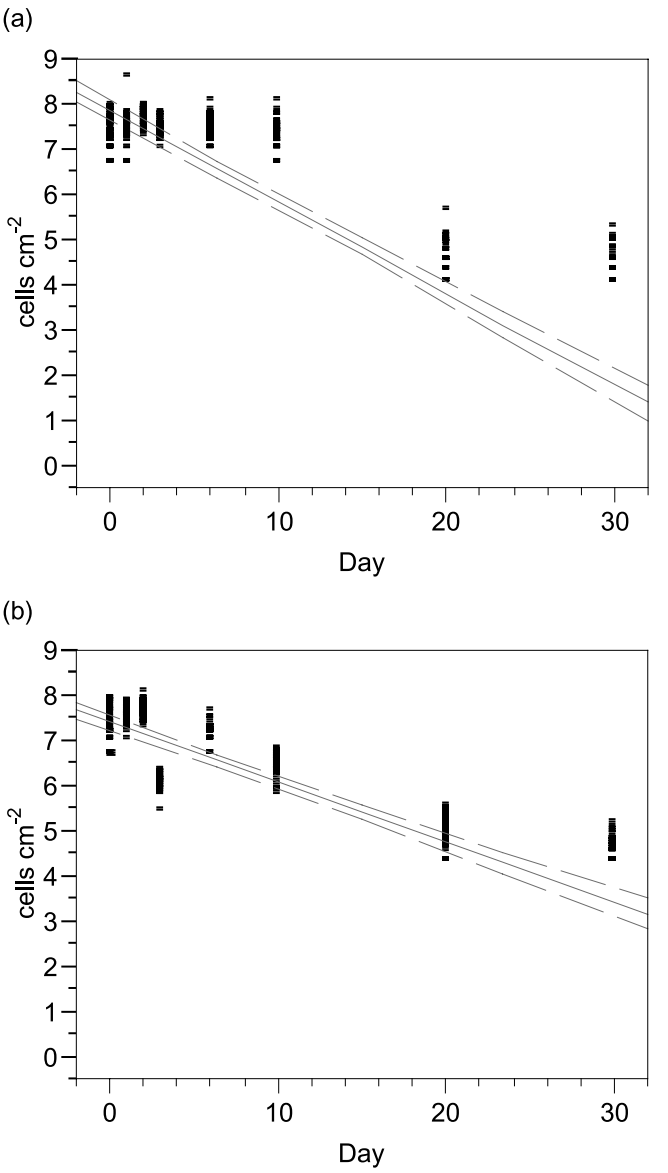

Figure 2 | Log-linearity between declining aeromonad numbers (cells cm^{-2}) on (a) GL coupons and (b) SS coupons over time (days) as determined by fluorescence *in situ* hybridization (FISH). (Line indicates best linear fit±one standard deviation.)

DISCUSSION

Quantification of aeromonads from different distribution systems

Despite a lack of conclusive evidence suggesting that the presence of *Aeromonas* in drinking water is directly attributed to outbreaks of disease, their potential health implications are gaining increasing interest (Szewzyk *et al.* 2000; WHO 2002; EPA 2003). Results from the present study showed that aeromonads were isolated from biofilms formed in potable and recycled water environments, thereby indicating their potential to colonize and subsequently compromise both systems. Results

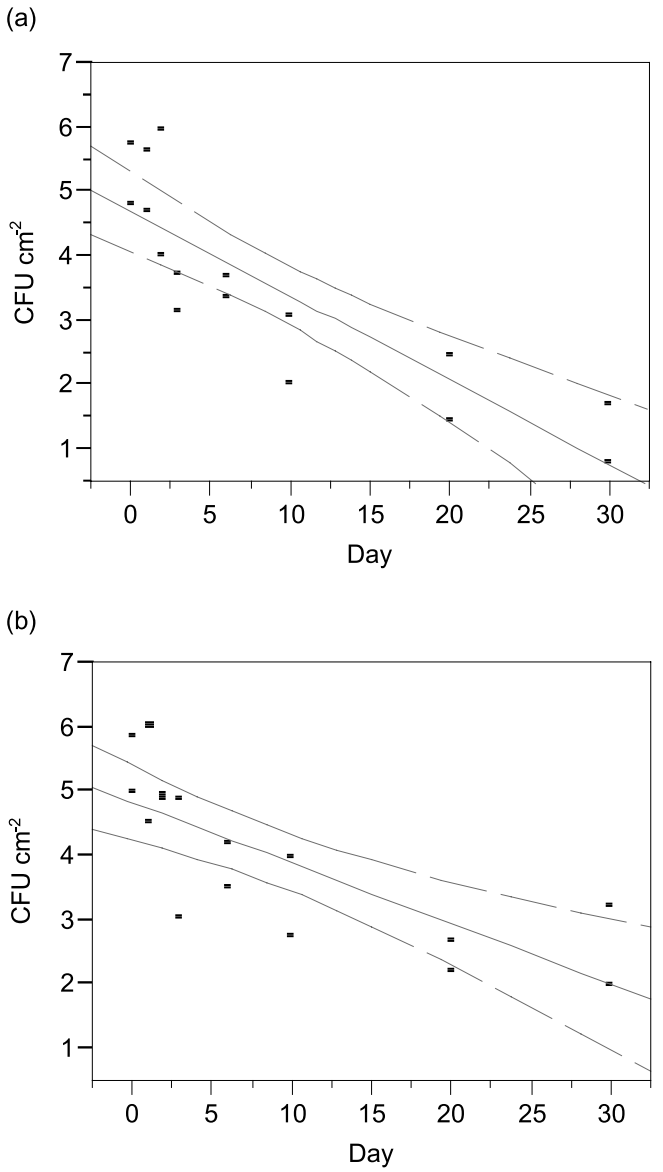

Figure 3 | Log-linearity between declining aeromonad numbers (CFU cm^{-2}) on (a) GL coupons and (b) SS coupons over time (days) as determined by culturing (Line indicates best linear fit±one standard deviation.)

obtained in this study are also consistent with other studies of drinking water distribution pipe biofilms where aeromonads have been detected (Holmes & Nicolls 1995; Walker *et al*. 1995; Szewzyk *et al*. 2000; Chauret *et al*. 2001).

Differences in the number of aeromonads were observed both between and within systems investigated. The total absence of aeromonads from the MRD-p may have been attributed to the higher mean hydraulic demand and shear ($Re = 7.6 \times 10^3$) compared with the MRD-r ($Re = 3.1 \times 10^3$) and BR™ ($Re = 6.6 \times 10^2$) (Storey 2002), given that all were serviced from the same water source. This finding warrants further investigations, as aeromonads are routinely isolated from potable water distribution pipe systems (Holmes & Nicolls 1995; Kuhn *et al*. 1997*b*) that are likely to encounter similar hydraulic conditions. Whilst there was no significant difference in chlorine concentration between the MRD-p, MRD-r and BR™ devices, a slightly elevated temperature observed within the MRD-r and BR™ may have contributed to the growth of aeromonads observed in this study. Increasing levels of *Aeromonas* in drinking water systems have been found to correlate with higher temperatures (Chauret *et al*. 2001), although this study referred to seasonal temperature patterns rather than the small elevations in temperatures we observed in our study.

The higher numbers of *Aeromonas* in the BR™ compared with the MRDs was consistent with a higher biofilm biomass in the BR™. Although *Aeromonas* are known to grow under nutrient-limited conditions (Van der Koij & Hijnen 1988; Walker *et al*. 1995), more biofilm biomass and greater nutrient availability is likely to favour an increased growth of this genus. This was also seen in the more nutrient-rich model recycled water distribution system (PL) where *Aeromonas* were detected on 100% of coupons investigated and the biofilm biomass was significantly higher than in devices supplied with drinking water.

Within the BR™ device, a significantly higher number of aeromonads was found on uPVC than on SS coupons. Assanta *et al*. (1998) also observed a higher number of *Aeromonas* cells on plastic substrata (polybutylene) compared with SS. Higher numbers of *Aeromonas* also correlated with higher numbers of HPC on uPVC than on SS. Other studies have, however, not found any apparent relationship between the presence of *Aeromonas* and HPC in distribution pipe biofilms (Gavriel *et al*. 1998; Chauret *et al*. 2001). Whilst there were minor differences in total protein and total carbohydrate concentrations between the two substrata, total numbers of bacteria were higher on SS than on uPVC (10^6 vs. 10^5 cells cm^{-2}, respectively).

Our total bacterial counts were, however, in the same range as reported in other studies of biofilms on SS and uPVC (10^4–10^6 cells cm^{-2}) (Pedersen 1990; Percival *et al*. 1998), although Pedersen (1990) did not find any differences in total bacterial counts on SS compared with PVC.

Significantly more culturable and total bacteria were observed on GL substrata compared with SS in the model-recycled water system (PL). Despite this, no significant differences were observed between *Aeromonas* numbers and substrata, confirming that a relationship between general bacterial counts and *Aeromonas* numbers might not exist (Gavriel *et al*. 1998; Chauret *et al*. 2001). The opposite was, however, observed in the BR™. Other studies on biofilm development in specific waters have not found significant differences in bacterial numbers on either glass or asphalt-coated mild steel, nor glass or Teflon (van der Kooij *et al*. 1995; Walker *et al.* 1995). The concentration of total protein and total carbohydrate was also inconsistent with the other biofilm-biomass results, indicating the need for further investigations of biofilm formation and substrata in specific waters.

Due to the highly heterogeneous nature of biofilm coverage on a substratum surface, microorganism numbers may be described in terms of the total amount of biofilm biomass covering the surface area of interest (Storey & Ashbolt 2002). Carbohydrates are traditionally thought to be the major component of biofilms (Ford *et al*. 1991; Lazarova & Manem 1995), although Storey & Ashbolt (2002) have shown that total protein content is a better index of biofilm biomass for the systems they examined. Results from the BR™ in our study showed that, by using normalized aeromonad numbers when comparing substrata (uPVC vs. SS), no significant difference in aeromonad numbers was observed, whereas the situation was opposite using un-normalized *Aeromonas* numbers. This indicates that the number of aeromonads is a function of the amount of biofilm biomass, which can also be dependent on the nature of the substrata. This confirms that biofilm biomass can be a useful measure of potential pathogen incorporation into biofilms, although discrimination between carbohydrate and protein as a better index of biomass could not be made in this study.

Identification of aeromonads

Of the aeromonad-positive isolates, none was identified through biochemical testing as being of human health significance (i.e. *A. hydrophila*, *A. caviae* or *A. veronii-sobria*) although PCR and FISH confirmed them as aeromonads. This lack of agreement between phenotypic and genotypic identification of aeromonads has also been reported elsewhere (Dorsch *et al*. 1994; Ashbolt *et al*. 1995). This anomaly may therefore indicate that many species of the genus *Aeromonas* have not yet been identified. This may be attributed to the complicated taxonomy of this genus with regard to species identification at both biochemical and molecular levels (Janda 1991; Millership 1996). The 70% agreement observed between PCR and FISH techniques for isolates from potable water systems could be explained in part by the fact that cells with very low ribosome number, which are often associated with oligotrophic potable water environments, were stained negative by FISH probing but positive by PCR. The slightly higher (76%) agreement between PCR and FISH for isolates from the more nutrient-rich recycled wastewater, however, may indicate that other factors are also involved, such as low target accessibility, ineffective probe labelling and non-optimal hybridization conditions (Amann *et al*. 1995). Furthermore, non-specific amplification and thereby an incorrect amplification of DNA can be a problem with PCR. Based on the results obtained in the current study, the disagreement between PCR and FISH methods indicates that the use of either technique alone could lead to loss of important information.

Integration and persistence of *A. hydrophila* in a biofilm environment

Challenging the recycled wastewater biofilms with a slug dose of *A. hydrophila* demonstrated that such a potential pathogen readily incorporates into a biofilm where it may remain infectious for prolonged periods of time. In a colonization study undertaken with *A. hydrophila* in drinking water biofilms, Walker *et al*. (1995) observed that *A. hydrophila* was incorporated into biofilms and could remain viable for a further 7 days. The extended longevity of *A. hydrophila* observed in our study indicates that a

more nutrient-rich environment such as that found in recycled wastewater can prolong the survival of introduced bacterial cells.

Detection of aeromonads by FISH in the pipe loop demonstrated a maxiumum of 3–4 log increase compared with those detected by standard culturing techniques. Towards the end of the experiment, 1-log higher aeromonad numbers were observed with FISH compared with culturing. The higher and more stable *A. hydrophila* numbers observed with the FISH method indicates that relatively high numbers of *Aeromonas* cells persisted in the biofilm even after the majority of cells had ceased to be culturable. Prolonged persistence of *A. hydrophila* in different water environments using direct count methods compared with culturing techniques has also been observed in other studies (Wai *et al*. 2000; Rahman *et al.* 2001; Mary *et al*. 2002). At the conclusion of the experiment (day 30), a significantly higher number of FISH-positive aeromonad cells was detected on SS coupons compared with the background noise found on day -1. This could indicate that a minor population of the introduced *A. hydrophila* managed to establish themselves within the biofilms.

Establishment of introduced bacteria within laboratory-grown biofilms has also been reported elsewhere (Stewart *et al*. 1997; Buswell *et al*. 1998). Consistent with the results obtained in our study, the work of Buswell *et al.* (1998) also showed that bacterial persistence within biofilms increased when bacteria were enumerated by direct staining techniques and not culture-based detection methods. The use of traditional plating techniques carries the inherent risk of underestimating bacterial numbers because of the widely appreciated limitations of methods that rely on the culturability of bacteria (Amann *et al*. 1995). To overcome such limitations, the application of molecular techniques such as FISH is invaluable. The human health significance of the more persistent aeromonads in a non-culturable form, however, still remains to be addressed in relation to the concept of active or viable but non-culturable (VBNC) states of bacteria. Several studies have demonstrated the existence of a VBNC state of *Aeromonas* in different waters, but the resuscitation from this state and possible public health significance is controversial and warrants further investigations (Allen-Austin *et al*. 1984; Rose *et al*. 1990; Morgan *et al*. 1991; Wai *et al*. 2000; Rahman *et al*. 2001; Mary *et al*. 2002).

Biofilm detachment, either by continuous erosion of small portions of biofilm or more rapid and massive loss of biofilm by sloughing (Characklis 1990), can contribute to bacterial contamination of the bulk water and thereby present a potential health risk to consumers. In our study, biofilm biomass remained relatively constant throughout the experimental period, indicating that loss of activity and culturability rather than erosion of biofilm from coupon surface accounts for the declining numbers of *A. hydrophila* cells. Declining numbers of aeromonad cells as detected with FISH, however, can also be explained in terms of lower ribosome (copy) numbers in the bacterial cells and thereby a lower hybridization signal. An attempt to treat coupons used for FISH with the probe active count (PAC) method described by Kalmbach *et al*. (1997) was therefore carried out. However it did not strengthen hybridization signals for the cells nor result in a higher detection of cells (results not shown). The role of erosion in the loss of cells therefore cannot be ruled out altogether, indicating that a minor degree of biofilm erosion may explain the decline of *A. hydrophila* numbers.

First-order kinetics did not adequately describe the behaviour of *A. hydrophila* observed in our study. The declining numbers could be explained in terms of a stable plateau or latency followed by a rapid decline that was further followed by a more constant, gradual decline. Such deviation from true first-order kinetics has also been observed in a study of the persistence of enteric virions within biofilms, which better fitted a bi-phasic pattern (Storey & Ashbolt 2001).

CONCLUSIONS

In summary, this study has shown that *Aeromonas* can incorporate and persist in biofilms in distribution pipe systems serviced with different water sources. Whilst the exact human health significance of this remains unknown, this phenomenon could imply a potentially increased concern for consumers of distribution pipe water, particularly

recycled waters, given their larger incidence of biofilm-producing nutrients as well as aeromonad numbers. Integration and persistence of *A. hydrophila* in biofilms was shown to deviate from conventional 1st order kinetic decay, suggesting their potential for longer persistence within a water distribution system. Traditional culturing techniques should be supported with molecular techniques such as PCR and FISH, for both species identification and enumeration purposes, given the often anomalous relationship observed between the two in this study.

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ABBREVIATIONS

BR™	Biofilm Reactor™
BSS	biofilm sampling site
FISH	fluorescence *in situ* hybridization
GL	glass
MRD	modified Robbins device
MRD-p	MRD in potable water
MRD-r	MRD in recycled water
PCR	polymerase chain reaction
PL	pipe loop
Re	Reynolds number
SS	stainless steel
uPVC	unplasticized polyvinyl chloride

REFERENCES

Allen-Austin, D., Austin, B. & Colwell, R. R. 1984 Survival of *Aeromonas salmonicida* in river water. *FEMS Microbiol. Lett.* **21**, 143–146.

Amann, R. I., Ludwig, W. & Schleifer, K. H. 1995 Phylogenetic identification and *in-situ* detection of individual microbial-cells without cultivation. *Microbiol. Rev.* **59**, 143–169.

Araujo, R. M., Arribas, R. M. & Pares, R. 1991 Distribution of *Aeromonas* species in waters with different levels of pollution. *J. Appl. Bacteriol.* **71**, 182–186.

Armon, R., Starosvetzky, J., Arbel, T. & Green, M. 1997 Survival of *Legionella pneumophila* and *Salmonella typhimurium* in biofilm systems. *Wat. Sci. Technol.* **35**(11-12), 293–300.

Ashbolt, N. J., Ball, A., Dorsch, M., Turner, C., Cox, P., Chapman, A. & Kirov, S. M. 1995 The identification and human health significance of environmental aeromonads. *Wat. Sci. Technol.* **31**(5–6), 263–269.

Assanta, M. A., Roy, D. & Montpetit, D. 1998 Adhesion of *Aeromonas hydrophila* to water distribution system pipes after different contact times. *J. Food Prot.* **61**, 1321–1329.

Baloda, S. B., Krovacek, K., Eriksson, L., Linne, T. & Mansson, I. 1995 Detection of aerolysin gene in *Aeromonas* strains isolated from drinking-water, fish and foods by the polymerase chain-reaction. *Comp. Immuno. Microbiol. Infect. Dis.* **18**, 17–26.

Borchardt, M. A. 2003 Aeromonas isolates from human diarrheic stool and groundwater compared by pulsed-field gel electrophoresis. *Emerging Infect. Dis.* **9**, 224–228.

Buswell, C. M., Herlihy, Y. M., Lawrence, L. M., McGuiggan, J. T. M., Marsh, P. D., Keevil, C. W. & Leach, S. A. 1998 Extended survival and persistence of *Campylobacter* spp. in water and aquatic biofilms and their detection by immunofluorescent-antibody and -rRNA staining. *Appl. Environ. Microbiol.* **64**, 733–741.

Characklis, W. G. 1990 Biofilm processes. In *Biofilms* (ed. W. G. Characklis & K. C. Marshall), pp. 195–231. John Wiley and Sons, Inc., New York.

Chauret, C., Volk, C., Creason, R., Jarosh, J., Robinson, J. & Warnes, C. 2001 Detection of *Aeromonas hydrophila* in a drinking-water distribution system: a field and pilot study. *Can. J. Microbiol.* **47**, 782–786.

Deodhar, L. P., Saraswathi, K. & Varudkar, A. 1991 *Aeromonas* spp and their association with human diarrheal disease. *J. Clin. Microbiol.* **29**, 853–856.

Dorsch, M. & Stackebrandt, E. 1992 Some modifications in the procedure of direct sequencing of PCR amplified 16S-rDNA. *J. Microbiol. Meth.* **16**, 271–279.

Dorsch, M., Ashbolt, N. J., Cox, P. T. & Goodman, A. E. 1994 Rapid identification of *Aeromonas* species using 16S rDNA targeted oligonucleotide primers–a molecular approach based on screening of environmental isolates. *J. Appl. Bacteriol.* **77**, 722–726.

EPA 2003 *Screening Survey for* Aeromonas *at Selected Public Water Systems*. Environmental Protection Agency, www.epa.gov.

Essers, B., Burnens, A. P., Lanfranchini, F. M., Somaruga, S. G. E., von Vigier, R. O., Schaad, U. B., Aebi, C. & Bianchetti, M. G. 2000 Acute community-acquired diarrhea requiring hospital admission in Swiss children. *Clin. Infect. Dis.* **31**, 192–196.

Ford, T., Sacco, E., Black, J., Kelley, T., Goodacre, R., Berkeley, R. C. W. & Mitchell, R. 1991 Characterization of exopolymers of aquatic bacteria by pyrolysis-mass spectrometry. *Appl. Environ. Microbiol.* **57**, 1595–1601.

Gavriel, A. A., Landre, J. P. B. & Lamb, A. J. 1998 Incidence of mesophilic *Aeromonas* within a public drinking water supply in north-east Scotland. *J. Appl. Microbiol.* **84**, 383–392.

Giovannoni, S. J., Delong, E. F., Olsen, G. J. & Pace, N. R. 1988 Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial-cells. *J. Bacteriol.* **170**, 720–726.

Gold, W. L. & Salit, I. E. 1993 *Aeromonas hydrophila* infections of skin and soft tissue: report of 11 cases and review. *Clin. Infect. Dis.* **16**, 69–74.

Havelaar, A. H., Schets, F. M., van Silfhout, A., Jansen, W. H., Wieten, G. & van der Kooij, D. 1992 Typing of *Aeromonas* strains from patients with diarrhoea and from drinking water. *J. Appl. Bacteriol.* **72**, 435–444.

Hazen, T. C., Fliermans, C. B., Hirsch, R. P. & Esch, G. W. 1978 Prevalence and distribution of *Aeromonas hydrophila* in the United States. *Appl. Environ. Microbiol.* **36**, 731–738.

Holmes, P. & Nicolls, L. M. 1995 Aeromonads in drinking-water supplies—their occurrence and significance. *J. Inst. Wat. Environ. Managmt* **9**, 464–469.

Janda, J. M. 1991 Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. *Clin. Microbiol. Rev.* **4**, 397–410.

Janda, J. M. & Abbot, S. L. 1998 Evolving concepts regarding the genus *Aeromonas*: An expanding panorama of species, disease presentations, and unanswered questions. *Clin. Infect. Dis.* **27**, 332–344.

Janda, J. M., Guthertz, L. S., Kokka, R. P. & Shimada, T. 1994 *Aeromonas* species in septicemia—laboratory characteristics and clinical observations. *Clin. Infect. Dis.* **19**, 77–83.

Kalmbach, S., Manz, W. & Szewzyk, U. 1997 Dynamics of biofilm formation in drinking water: Phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and *in situ* hybridization. *FEMS Microbiol. Ecol.* **22**, 265–279.

Kampfer, P., Erhart, R., Beimfohr, C., Bohringer, J., Wagner, M. & Amann, R. 1996 Characterization of bacterial communities from activated sludge: Culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA- targeted oligonucleotide probes. *Microb. Ecol.* **32**, 101–121.

Kühn, I., Allestam, G., Huys, G., Janssen, P., Kersters, K., Krovacek, K. & Stenström, T.-A. 1997*a* Diversity, persistence, and virulence of *Aeromonas* strains isolated from drinking water distribution systems in Sweden. *Appl. Environ. Microbiol.* **63**, 2708–2715.

Kühn, I., Huys, G., Coopman, R., Kersters, K. & Janssen, P. 1997*b* A 4-year study of the diversity and persistence of coliforms and *Aeromonas* in the water of a Swedish drinking water well. *Can. J. Microbiol.* **43**, 9–16.

Lazarova, V. & Manem, J. 1995 Biofilm characterization and activity analysis in water and waste-water treatment. *Wat. Res.* **29**, 2227–2245.

Lunder, T. & Håstein, T. 1990 Infeksjoner med *Aeromonas salmonicida*. In *Fiskehelse—sykdommer, behandling, forebygging* (ed. T. T. Poppe), pp. 140–146. John Grieg Forlag AS (in Norwegian).

Mackay, W. G., Gribbon, L. T., Barer, M. R. & Reid, D. C. 1998 Biofilms in drinking water systems—a possible reservoir for *Helicobacter pylori*. *Wat. Sci. Technol.* **38**(12), 181–185.

Manz, W., Amann, R., Ludwig, W., Wagner, M. & Schleifer, K. H. 1992 Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria—problems and solutions. *System Appl. Microbiol.* **15**, 593–600.

Mary, P., Chihib, N. E., Charafeddine, O., Defives, C. & Hornez, J. P. 2002 Starvation survival and viable but nonculturable states in *Aeromonas hydrophila*. *Microb. Ecol.* **43**, 250–258.

Millership, S. E. 1996 Identification. In *The Genus Aeromonas* (eds. Austin B., Altwegg, M., Gosling, P. J. & Joseph, S. W.), pp. 85–107. John Wiley & Sons, Chichester.

Morgan, J. A. W., Cranwell, P. A. & Pickup, R. W. 1991 Survival of *Aeromonas salmonicida* in lake water. *Appl. Environ. Microbiol.* **57**, 1777–1782.

Nakano, H., Kameyama, T., Venkateswaran, K., Kawakami, H. & Hashimoto, H. 1990 Distribution and characterization of hemolytic and enteropathogenic motile *Aeromonas* in aquatic environments. *Microbiol. Immunol.* **34**, 447–458.

Pedersen, K. 1990 Biofilm development on stainless steel and PVC surfaces in drinking water. *Wat. Res.* **24**, 239–243.

Percival, S. L. & Walker, J. T. 1999 Potable water and biofilms: A review of the public health implications. *Biofouling* **14**, 99–115.

Percival, S. L., Knapp, J. S., Edyvean, R. & Wales, D. S. 1998 Biofilm development on stainless steel in mains water. *Wat. Res.* **32**, 243–253.

Poffe, R. & Op De Beeck, E. 1991 Enumeration of *Aeromonas hydrophila* from domestic waste-water treatment plants and surface waters. *J. Appl. Bacteriol.* **71**, 366–370.

Popoff, M. & Veron, M. 1976 A taxonomic study of the *Aeromonas hydrophila–Aeromonas punctata* group. *J. Gen. Microbiol.* **94**, 11–22.

Rahman, M. H., Suzuki, S. & Kawai, K. 2001 Formation of viable but non-culturable state (VBNC) of *Aeromonas hydrophila* and its virulence to goldfish, *Carassius auratus*. *Microbiol. Res.* **156**, 103–106.

Rippey, S. R. & Cabelli, V. J. 1985 Growth characteristics of *Aeromonas hydrophila* in limnetic waters of varying trophic state. *Arch. Hydrobiol.* **104**, 311–319.

Rose, A. S., Ellis, A. E. & Munro, A. L. S. 1990 The survival of *Aeromonas salmonicida* subsp. *salmonicida* in sea water. *J. Fish Dis.* **13**, 205–214.

Stewart, P. S., Camper, A. K., Handran, S. D., Huang, C. T. & Warnecke, M. 1997 Spatial distribution and coexistence of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in biofilms. *Microb. Ecol.* **33**, 2–10.

Storey, M. V. 2002 The ecology of enteric viruses within distribution pipe biofilms. PhD Thesis, University of New South Wales, Australia.

Storey, M. V. & Ashbolt, N. J. 2001 Persistence of two model enteric viruses (B40-8 and MS-2 bacteriophages) in water distribution pipe biofilms. *Wat. Sci. Technol.* **43**(12), 133–138.

Storey, M. V. & Ashbolt, N. J. 2002 A comparison of methods and models for the analysis of distribution pipe biofilms. *Wat. Sci. Technol.: Wat. Suppl.* **2**(4), 73–80.

Szewzyk, U., Manz, W., Amann, R. I., Schleifer, K. H. & Stenström, T- A. 1994 Growth and *in situ* detection of a pathogenic *Escherichia coli* in biofilms of a heterotrophic water-bacterium by use of 16S- and 23S-rRNA directed fluorescent oligonucleotide probes. *FEMS Microbiol. Ecol.* **13**, 169–176.

Szewzyk, U., Szewzyk, R., Manz, W. & Schleifer, K. H. 2000 Microbiological safety of drinking water. *Ann. Rev. Microbiol.* **54**, 81–127.

Van Der Kooij, D. & Hijnen, W. A. M. 1988 Nutritional versatility and growth-kinetics of an *Aeromonas hydrophila* strain isolated from drinking-water. *Appl. Environ. Microbiol.* **54**, 2842–2851.

Van Der Kooij, D., Veenendaal, H. R., Baars-Lorist, C., van der Klift, D. W. & Drost, Y. C. 1995 Biofilm formation on surfaces of glass and teflon exposed to treated water. *Wat. Res.* **29**, 1655–1662.

Wai, S. N., Mizunoe, Y., Takade, A. & Yoshida, S. 2000 A comparison of solid and liquid media for resuscitation of starvation—and low-temperature-induced nonculturable cells of *Aeromonas hydrophila*. *Arch. Microbiol.* **173**, 307–310.

Walker, J. T., Mackerness, C. W., Rogers, J. & Keevil, C. W. 1995 Heterogenous mosaic biofilm—a haven for waterborne pathogens. In *Microbial Biofilms* (ed. H. M. Lappin-Scott & J. W. Costerton), pp. 196–204. Cambridge University Press, Cambridge.

WHO 2002 *Guidelines for Drinking-water Quality. Addendum: Microbiological Agents in Drinking-water*, 2nd edition. World Health Organization, Geneva.