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Survival and nutritional requirements of three bacteria isolated from ultrapure water

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Bacteria isolated previously from ultrapure water (UPW) systems were examined for their ability to survive in UPW, with the ultimate goal of elucidating potential carbon and energy sources for the bacteria. Two strains of *Ralstonia pickettii* isolated from different areas within the UPW system (pretreatment and polishing loop, and referred to as strains 3A1 and MF254A, respectively) and a strain of *Bradyrhizobium* sp. were compared to increase our understanding of the fundamental behavior of bacteria contaminating UPW. *R. pickettii* (3A1) grew significantly slower in R2A medium, with a final cell yield much lower than the isolate from the polishing loop. In addition, *R. pickettii* MF254A showed a broader substrate range than either strain 3A1 or *Bradyrhizobium* sp. In UPW, there appears to be a threshold cell concentration (approximately 10^6 colony-forming units/ml), whereby the cell numbers remain constant for a prolonged period of 6 months or more. Below this concentration, rapid proliferation is observed until the threshold concentration is attained. Preliminary experiments suggested that nitrogen gas (frequently added to UPW storage tanks) may contribute to growth of *Bradyrhizobium* sp. Above the threshold concentration, the strain of *Ralstonia* sp. isolated from the polishing loop was capable of cryptic growth with heat-killed cells in UPW. However, cryptic growth was not observed when the cells supplied as nutrients were killed using UV254 light. Furthermore, cryptic growth did not appear to contribute significantly to proliferation of *Bradyrhizobium* sp. or *Ralstonia* sp. 3A1 (isolated from the pretreatment loop). We believe that cryptic growth may aid survival of the bacteria in UPW, but further experiments are warranted to prove this phenomenon conclusively.
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Introduction

Ultrapure water (UPW) is essential to many industries, particularly the semiconductor and pharmaceutical industries, where the presence of a single bacterial cell can be detrimental to the quality of the final product [18,39]. UPW production incorporates both pretreatment and polishing stages to remove organics and inorganics, but producing UPW devoid of bacterial contamination remains one of the biggest challenges facing UPW industries. Once bacteria are present in the system, they can affect practically every component in the UPW system, including an increase in frictional and heat transfer resistance [6,16,27], biofouling of reverse osmosis membranes [33,34], grow-through of filters [9,20,24] and colonization of pipelines [30], ultimately leading to contamination of the distribution line.

Although UPW can be considered an extreme environment, a group of bacteria called oligotrophs is capable of growth on very low concentrations of nutrients, and consequently can thrive in UPW [31]. They survive by adhering to the inner piping, resulting in formation of a biofilm. In aqueous environments, oligotrophs tend to produce extracellular polysaccharides (EPS), which act both as a "sticky glue" to hold the biofilm together, as well as concentrate nutrients derived from the flowing water or products of cellular metabolism [10–12].

Although there are reports describing the ability of bacteria to survive and proliferate in distilled water [8,14,15,19], there is an apparent lack of knowledge regarding the survival of bacteria in UPW. In previous reports [23,26], we described the diversity of bacteria found in industrial UPW systems and illustrated that, generally, *Ralstonia pickettii* and *Bradyrhizobium* sp. appear ubiquitous to UPW systems (with *R. pickettii* present in most of the analyzed UPW systems), irrespective of location. Further, we showed that a significant percentage (50–90%) of the bacterial cells enumerated by direct staining and epifluorescence microscopy were nonviable [26].

The influence of dead bacterial cells in UPW has been somewhat overlooked, partially due to the current industry standards that recommend plating techniques for bacterial enumeration [2,3]. However, some bacteria can utilize the lysis products from dead biomass as carbon and energy sources, a phenomenon known as cryptic growth [35]. Although it is unclear if all bacteria are capable of cryptic growth, it seems likely that oligotrophs possess this characteristic, which allows them to survive in a feast or famine environment. Under the extreme conditions of UPW, oligotrophs are likely to utilize whatever energy resources become available, or face starvation.

UPW systems typically consist of a make-up portion (for treatment of the incoming water) and a polishing loop (to produce UPW), with the UPW delivered to the user *via* a distribution loop (Figure 1). Current industrial standards specify that UPW should

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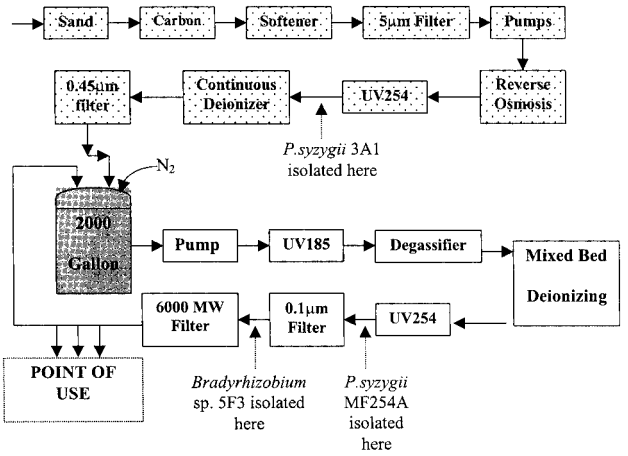

Figure 1 Schematic of the UPW system (pilot scale, University of Arizona) used in this study. The areas of isolation for the bacterial strains used in this study are indicated. (⊡) Refers to the pretreatment stages; (□) indicates the polishing loop.

have a total organic carbon (TOC) content of less than 1 ppb, a particle count (greater than 0.05 μm in size) of less than 500 l^{-1} and an oxygen content of less than 1 ppb. The bacterial bioburden should not exceed 1–10 colony-forming units (CFU)/l, and it is emphasized that this refers only to viable bacterial cells [3].

With respect to bacterial contamination, there is an obvious need for more efficient quality control of UPW systems to minimize costly, unscheduled shutdowns. However, to date, little is known regarding the ability of bacteria in UPW to persist and recolonize areas. Therefore, the aim of the present work was to test the hypothesis that bacteria contaminating UPW have adapted to a unique, oligotrophic niche. This was accomplished by comparing two strains of *R. pickettii*: one isolated from the pretreatment and one from the polishing loop of an UPW system. Furthermore, we have investigated the fundamental behavior and growth kinetics of *R. pickettii* and *Bradyrhizobium* sp. in UPW. Information gained regarding potential sources of carbon and energy for the contaminating bacteria may assist UPW users in implementing additional bacterial control measures. We report here on the influence of cryptic growth in such an environment as well.

Materials and methods

Bacteria and growth conditions

Three bacterial strains previously isolated from UPW were employed for the purposes of this study. The first isolate, a strain of *R. pickettii*, was isolated from the post-UV254 area within the polishing loop and was designated *R. pickettii* MF254A (Figure 1). A second strain of *R. pickettii* (designated *R. pickettii* 3A1) was isolated from the pretreatment loop after UV254 irradiation and was included for comparison to *R. pickettii* MF254A. The third bacterium of interest, a *Bradyrhizobium* sp. (designated strain 5F3), was isolated from the area after the 0.1-μm filter. This bacterium contains *nifH* genes [23], suggesting that it may be capable of nitrogen fixation. All strains were previously identified on the basis of their 16*S* rRNA gene sequences [23] and were grown in R2A or diluted R2A, as appropriate [32]. Incubation was conducted at 25°C with aeration on an orbital shaker (180 rpm).

To determine potential growth substrates for the bacteria, a second medium, referred to as oligotrophic medium (OM), was developed and contained the following components (g/l): NaCl, 0.2; KCl, 0.2; Na_2HPO_4, 0.2; NH_4NO_3, 0.1; $MgCl_2{\cdot}6H_2O$, 0.1; NH_4Cl, 0.05; $(NH_4)_2SO_4$, 0.02 (pH~6.8). $MgCl_2{\cdot}6H_2O$ and $(NH_4)_2SO_4$ were prepared as 1000× stock solutions and sterilized separately to prevent precipitation within the medium. When required, the medium was buffered with 50 mM Na_2HPO_4–NaH_2PO_4 to give a final pH of 7.0. Substrates were added at a concentration of 0.25 g/l and growth was monitored by optical density (OD) measurements at 600 nm. The one exception was when humic acid was used as a growth substrate; due to intense coloration, growth was followed by regular plate counts (on R2A medium).

Survival of bacteria in UPW

Bacterial strains were grown in R2A to late exponential phase (OD_{600}~1.0, 0.6 and 0.7 for *R. pickettii* MF254A, *Bradyrhizobium* sp. and *R. pickettii* 3A1, respectively), whereupon they were harvested by centrifugation (8000 rpm, 15 min). The cells were washed three times in 10 mM Na_2HPO_4–NaH_2PO_4 (pH 7.0), before adding double filter-sterilized UPW to obtain the desired concentration of cells. Experiments were conducted over a range of cell concentrations, typically 10^1, 10^2, 10^3, 10^5 and 10^7 CFU/ml. The cells were then incubated overnight (with aeration on an orbital shaker at 180 rpm) at 25°C to induce starvation and deplete any remaining nutrients in the cell suspension. The following day, the cells were transferred directly to inoculate 1000-ml screw-top

Table 1 Substrate range for bacteria isolated from an UPW system

Substrate	*R. pickettii* MF254A	*R. pickettii* 3A1	*Bradyrhizobium* sp. 5F3
L-alanine	+	+	+
L-valine	+	+	–
L-cysteine	∓	–	–
L-leucine	+	+	–
D-L-tryptophan	–	∓	
L-isoleucine	+*	+*	–
D-L-serine	+	+	∓*
L-threonine	+	∓	–
L-lysine	–	∓	–
L-arginine	–	+	+
L-aspartic acid	+	∓	–
L-asparagine	+	+	∓
L-proline	+	∓	+
D-L-phenylalanine	+	+	–
Ammonium acetate	+	∓	+
Sodium pyruvate	+	∓	+
Sodium succinate	+	–	+
Sodium citrate	+	+	∓
Sodium tartrate	+	–	∓
L-arabinose	+	+	+
Galactose	+	+	+*
Mannose	+*	+	–
Cellobiose	+*	+	–
Rhamnose	+*	+*	∓*
Ribose	+*	+*	∓*
Glucosamine	+*	+	–
D-gluconic acid	+	+	+
Glutamine	+	∓	+
Xylose	+	+	+*
Humic acid	–	–	–

+$OD_{600} \geq 0.3$; ∓$0.1 \leq OD_{600} \leq 0.29$; –$OD_{600} < 0.1$.
*Extended lag phase (i.e., >50 h).

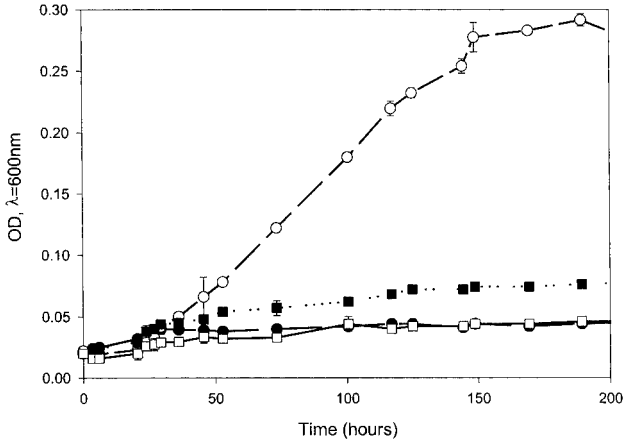

Figure 2 Growth of *Bradyrhizobium* sp. 5F3 under different conditions. The bacteria were grown in an oligotrophic medium (OM, pH 7.0) with sodium succinate as the sole carbon and energy source (0.5 g/l). The following growth conditions were tested: OM with no nitrogen source (closed circles, ●); OM with nitrogen source in the media (open circles, ○); OM with no nitrogen source in media but gaseous nitrogen blanket (closed squares, ■); and OM with no nitrogen source in media but gaseous argon blanket (open squares, □). Growth was monitored by OD measurements at 600 nm.

flasks (acid-washed) of double filter-sterilized UPW, and samples were taken to monitor viable count and total protein concentration.

Viable counts were determined by serial dilutions and plating onto R2A media. Growth of *R. pickettii* and *Bradyrhizobium* sp. was analyzed after incubation at 25°C for 5 and 10 days, respectively. The total protein concentration of the bacteria was determined by the BCA assay (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL). Briefly, the cells were washed once in buffer and boiled in 1 M NaOH for 10 min before centrifuging (12,000 rpm, 5 min) to remove cell debris. Total protein analysis was then conducted on the supernatant. The standards were also prepared in 1 M NaOH to prevent the need for neutralization of the samples. All experiments were completed in duplicate.

Effect of different environments on growth of the bacteria

To determine if the bacteria could fix nitrogen from its growth environment, *Bradyrhizobium* sp. and *R. pickettii* MF254A were grown in R2A to late exponential phase, then harvested and washed as described previously. After leaving the cell suspensions overnight to deplete nutrients, they were used to inoculate acid-washed, screw-top flasks containing OM, some of which were prepared without any nitrogen sources. Sodium succinate was supplied as the sole carbon and energy source at a concentration of 0.5 g/l. Several different growth environments were investigated, namely OM without a nitrogen source, OM with a nitrogen source in media, OM without a nitrogen source in media (but blanketed with gaseous nitrogen) and OM without a nitrogen source in media (but blanketed with gaseous argon). Experiments were conducted in duplicate and growth was monitored by OD measurements at 600 nm.

Determination of cryptic growth

Each bacterial strain was grown in duplicate in R2A and harvested by centrifugation as described above. Following the third wash, the cells were resuspended in double filter-sterilized UPW to the desired concentration. The cells to be used as the viable inoculum were left to starve at 25°C overnight, whereas the cells to be killed were either heat- or UV254-treated before also being transferred to the shaker until use.

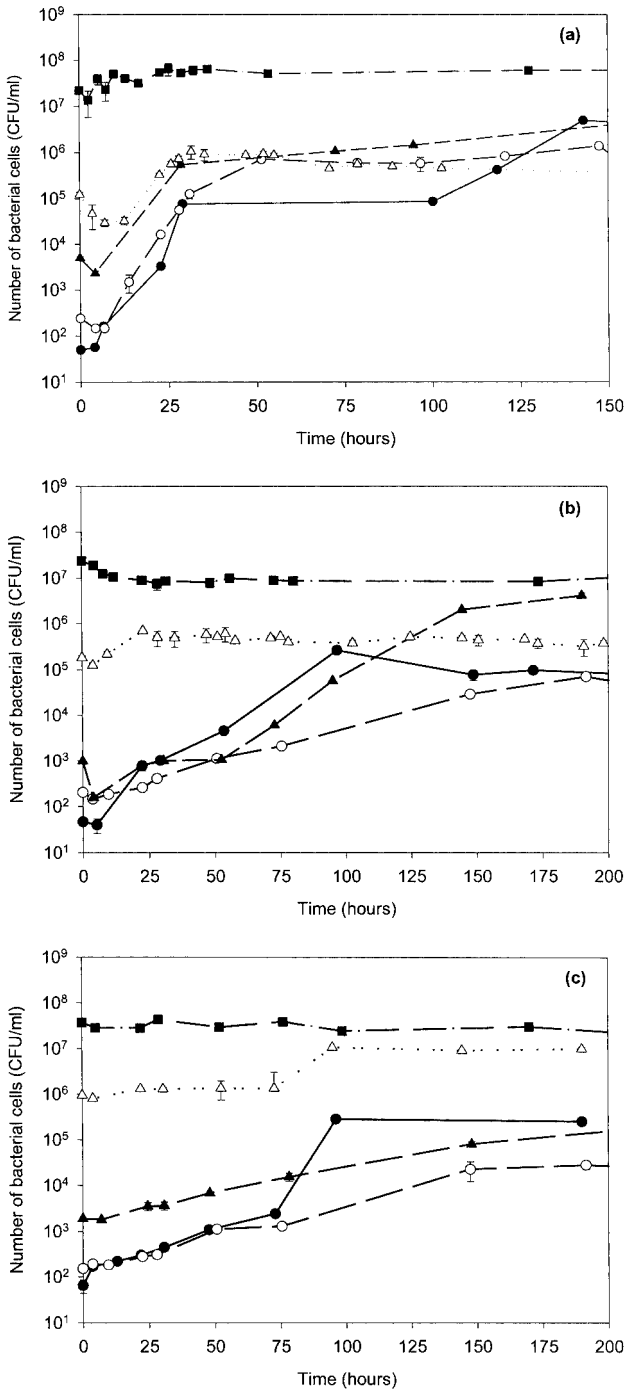

Figure 3 Growth and survival of (a) *R. pickettii* MF254A, (b) *Bradyrhizobium* sp. 5F3 and (c) *R. pickettii* 3A1 in UPW at 25°C. The effects of different initial cell concentrations were investigated: 50 (●), 200 (○), 5000 (▲), 10^5 (△) and 10^7 (■) CFU/ml. All UPW was double filter-sterilized prior to inoculation and had a TOC less than 2 ppb. Growth was monitored by plate counts on R2A medium.

Cells that were heat-killed were autoclaved at 121°C for 15 min. UV254-killed cells were exposed to UV254 (55 μW/cm^2 UV intensity; Atlantic Ultraviolet, Hauppage, NY) with continuous stirring for 20 min. Trials run prior to these experiments indicated that both forms of treatment were sufficient to kill the cells. The dead cells were then added to a screw-top, acid-washed flask containing 200 ml of double filter-sterilized UPW. Following inoculation of viable cells, the flasks were incubated at 180 rpm (25°C) and samples were obtained to monitor viable cells (CFU) and total protein. Each experiment was completed in duplicate. Control flasks were also included and comprised of UPW with the viable inoculum only, and UPW with dead cells only.

It should be emphasized that all UPW used in these studies was collected from the distribution loop of an UPW system (University of Arizona) and double filter-sterilized prior to use. The TOC prior to filtration was consistently in the range of 0.5–2 ppb.

Results

Comparison of R. Pickettii strains isolated from different sections of an UPW system

A number of bacterial strains isolated from various areas of the UPW system were identified as *R. pickettii* (and *Bradyrhizobium* sp.). However, the strains referred to as *R. pickettii* MF254A, *R. pickettii* 3A1 and *Bradyrhizobium* sp. 5F3 were selected as typical representatives of the contaminating microflora.

Both MF254A and 3A1 were identified as strains of *R. pickettii* (their 16*S* rRNA genes showed 99% homology to *R. pickettii*, ATCC49543T, as described in an earlier report) [23]. However, in view of their different areas of isolation within the system, we tested the hypothesis that strain MF254A (isolated from the polishing loop) may have adapted to the harsh UPW environment, and thus shows an increased extent of oligotrophy in comparison to strain 3A1 (isolated from the pretreatment loop).

When grown in R2A, strain MF254A grew significantly faster than strain 3A1 (0.27 and 0.033 h^{-1}, respectively). In addition, the final cell density of strain 3A1 was considerably lower than MF254A (approximately 1.9×10^7 and 3.2×10^9 CFU/ml, respectively). Interestingly, whilst strain MF254A could form colonies on diluted R2A (to 1:10,000 dilution), strain 3A1 could only grow in a 1:10 dilution and failed to grow on higher dilutions, implying that it was less oligotrophic than strain MF254A.

Differences were also noted between the two strains when grown on single carbon and energy sources (Table 1). Generally, strain MF254A grew on a wider range of substrates and achieved a higher cell yield than 3A1, which compares favorably with the broad substrate range believed to be characteristic of oligotrophs [31]. Most sugars could be used as energy sources, though a lag phase exceeding 50 h was often observed. The substrate range of *Bradyrhizobium* sp. 5F3 was also tested. Generally, this microorganism grew on fewer carbon sources, with significant growth on substrates supplying the organism with both carbon and nitrogen. None of the bacterial isolates was able to grow on L-methionine, nicotinic acid, thiamine, diaminopimelic acid or isopropyl alcohol.

Effects of different environments on growth of the bacteria

To investigate if *R. pickettii* MF254A and *Bradyrhizobium* sp. could grow when the only source of nitrogen was supplied as a gaseous blanket, each strain was inoculated into OM under various conditions. The results from this experiment are depicted in Figure 2.

Optimum growth of *Bradyrhizobium* sp. occurred when the nitrogen source was included in the growth media ($\mu\sim0.02$ h^{-1}). However, when the only nitrogen source was supplied in gaseous form, some growth was observed, albeit at a significantly slower rate ($\mu\sim0.007$ h^{-1}). In contrast, omitting nitrates from the OM, or blanketing the media with argon gas appeared to repress growth of this strain. This suggests that *Bradyrhizobium* sp. may be fixing nitrogen in this environment.

In contrast, *R. pickettii* MF254A grew only when nitrates were supplied in the OM and failed to grow under any other conditions tested (data not shown). Previously, we were unable to detect *nifH* genes in this strain [23] which, in agreement with these growth data, suggests that nitrogen fixation is probably not ongoing in this bacterium.

Survival of bacteria in UPW

At initial cell concentrations of 10^5 CFU/ml or less, all of the bacterial strains tested showed a remarkable ability to survive and increase their cell numbers significantly following inoculation into UPW (Figure 3a–c). Following the exponential growth period, the bacteria entered stationary phase, whereupon the cell numbers (CFU) stabilized, reaching a plateau, which was generally maintained thereafter. Indeed, some cultures were left for up to 6 months with no significant change in cell concentration.

The specific growth rates for each of the bacterial strains in UPW are shown in Table 2. It is noteworthy that the specific growth rates in UPW for all the bacterial strains tested (initial cell concentration of 10^5 CFU/ml or less) were similar to those determined in R2A. There was an approximate 10-fold difference in specific growth rate and final cell yield between the two strains of *R. pickettii*, supporting the proposal that *R. pickettii* MF254A (from the polishing loop) has adapted to a more oligotrophic existence than strain 3A1. *Bradyrhizobium* sp. also attained higher final cell densities than *R. pickettii* strain 3A1, and this probably also reflects its oligotrophic abilities.

Table 2 Specific growth rates (h^{-1}) for each of the bacterial strains in UPW

Initial cell concentration (CFU/ml)	*R. pickettii* MF254A	*R. pickettii* 3A1	Bradyrhizobium sp. 5F3
$\leq10^5$	0.22–0.28	0.03–0.04	0.04–0.10
$\geq10^6$	0.01–0.03	D	D
μ (in R2A)	0.27	0.03	0.11

D, decrease in cell concentration; CFU, colony-forming unit. All data were obtained after incubation at 25°C, and each value is the mean of duplicate experiments.

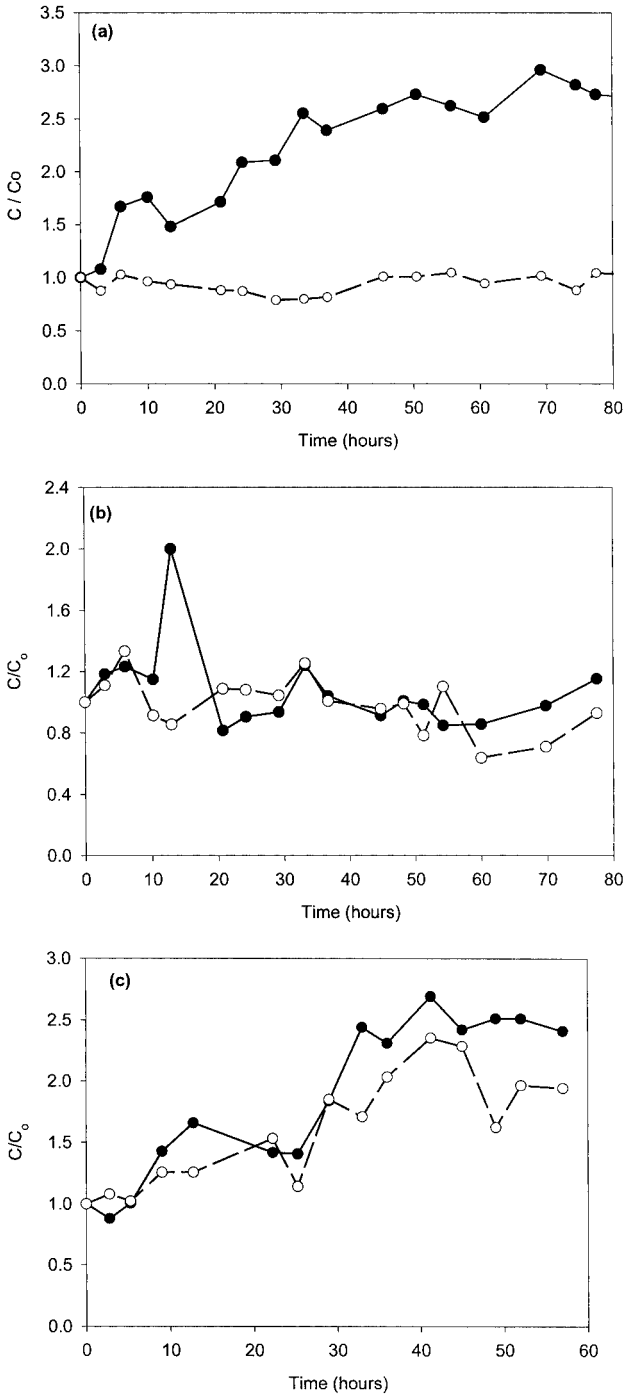

Figure 4 Influence of adding heat-killed cells to viable populations of (a) *R. pickettii* MF254A, (b) *R. pickettii* 3A1 and (c) *Bradyrhizobium* sp. 5F3 suspended in UPW at 25°C. Closed circles represent flasks to which the heat-killed cells were added, and the open circles represent the control flasks with only the viable bacterial suspension. Growth was monitored by OD and plate counts on R2A medium.

Under all cell concentrations and bacterial strains tested, the total protein concentration showed a very similar trend to that of the viable counts (data not shown). This implies that active proliferation was occurring using an unknown energy source, which provided both carbon and nitrogen for protein synthesis.

Determination of cryptic growth

Heat-killed cells: An increase in cell numbers and protein concentration was clearly observed following the addition of heat-killed cells to flasks containing UPW and the viable bacterial suspension of *R. pickettii* MF254A (Figure 4a). Growth occurred during the first 50 h (μ=0.02 h^{-1}) and the cell concentration increased correspondingly from 5.6×10^7 to 1.5×10^8 CFU/ml. This was not observed in the control when dead cells were omitted from the experiment (initial μ=0.0003 h^{-1}, followed by a decrease in cell concentration).

By comparison, the influence of cryptic growth in *R. pickettii* 3A1 was less apparent (Figure 4b). Upon addition of the dead cells, the viable cell count did not change significantly over the first 10 h (compared to the control). However, over the period of 10–20 h, the cell numbers approximately doubled (from 1.4×10^8 to 2.5×10^8 CFU/ml), which was not observed in the control. The cell concentration then declined to approximately 1.2×10^8 CFU/ml and did not change significantly for the remainder of the experiment.

Figure 4c depicts the growth of *Bradyrhizobium* sp. following addition of dead cells to the suspension. Growth of this strain was not significantly changed by the presence of the dead cells (approximately 0.07 h^{-1} compared to μ=0.05 h^{-1} for the control flask). However, a slightly higher cell concentration was achieved by adding the dead cells (2.7×10^7 CFU/ml compared to 2.0×10^7 CFU/ml in the absence of dead cells), suggesting that some nutrients could be utilized from the dead cells. All control experiments with dead cells only resulted in no growth.

UV254-killed cells: We have previously observed that areas immediately downstream of UV254 irradiation appear more prone to biofouling than preexposure areas [26]. We considered that this may be due to the influence of cryptic growth. To expand, UV-affected cells could die and lyse, providing the surviving bacteria (i.e., those shielded from UV irradiation by EPS, other cells or particulate matter) with a carbon and energy source to allow recolonization of the pipeline. However, even after incubation for 1 week, the cell concentrations of flasks containing the viable and UV-treated dead cells were not significantly different from the flasks containing only the viable cells (data not shown).

Discussion

A number of stages are incorporated into UPW production, some of which are specifically designed to prevent microbial contamination. Despite these measures, the occurrence of *R. pickettii* and *Bradyrhizobium* sp. has generally been shown as ubiquitous to UPW systems on a worldwide basis [23]. The mechanisms behind their persistence and propagation in UPW remain unclear. This study was, therefore, undertaken with the aim of investigating their survival in UPW and elucidating potential carbon and energy sources for the bacteria.

We previously discovered that both *R. pickettii* and *Bradyrhizobium* sp. were present in the pretreatment as well as the polishing loop [26] and, therefore, considered that the incoming water feeding the system was a potential source of contamination. Based on this fact, it seems reasonable to speculate that the bacteria present in the polishing loop must have adapted extensively to

allow them to survive and proliferate in the corrosive and extremely oligotrophic UPW environment.

By comparing *R. pickettii* strains 3A1 (from the pretreatment loop) and MF254A (from the polishing loop), we were able to investigate this hypothesis more extensively. Of particular interest was the significantly slower growth rate of *R. pickettii* strain 3A1 in R2A compared to *R. pickettii* MF254A. Furthermore, the inability of strain 3A1 to grow on media with a low nutrient content (i.e., greater than 1:10 dilutions of R2A) implies that this isolate does not possess oligotrophic characteristics to the same extent as *R. pickettii* MF254A. Since water in the pretreatment loop contains a higher TOC (particularly post reverse osmosis [17]), the increased copiotrophic characteristics of *R. pickettii* 3A1 are not an unexpected result.

Results from the comparison of strains MF254A and 3A1 have important implications. Since the area of colonization within the UPW system appears to influence the behavior and characteristics of the bacteria, different modes of removal may be required. Our data suggest that strains of *R. pickettii* from the polishing loop are clearly different from those found in the pretreatment loop. We propose that either the bacteria contaminating the polishing loop were introduced to the system by a different source than those in the pretreatment loop, or the bacteria are from the same original source, but significant evolution/adaptation has occurred. Further genomic comparison of these strains would help determine the most likely answer.

Examination of the substrate range for both *Ralstonia* strains showed that *R. pickettii* 3A1 utilized a narrower substrate range than strain MF254A and generally demonstrated a lower cellular yield on many of the substrates that allowed strain MF254A to grow rapidly. In agreement with Akagi and Taga [1] and Kim *et al* [22], we suggest that this may be an adapted survival strategy for oligotrophs in extreme environments.

Bradyrhizobium sp. demonstrated a narrower substrate range, but generally was capable of growth on most of the simple carbohydrates tested and some amino acids. These results are similar to those documented by Wagner *et al* [37], who found that strains of *Bradyrhizobium* preferentially grew on hexoses and pentoses, with no growth on disaccharides or nicotinic acid. It is important to note that in our experiments, *Bradyrhizobium* sp. 5F3 often required up to 8 days of incubation to demonstrate an appreciable increase in OD, an observation also supported by Wagner and Skipper [36], who suggested that *Bradyrhizobium* sp. should be incubated for at least 14 days.

Humic acid was not used by any of the strains tested over a range of concentrations. Humic acid is believed to contribute to the organic content of UPW, and we therefore considered that the microorganisms may be able to degrade (or partially degrade) the compound, possibly as a result of preexposure. However, humic acid has a complex structure, comprised of a partially aromatic structure with volatile aromatic compounds dispersed in the polymeric network. Therefore, degradation of this compound may be energetically unfavorable for these bacteria when it is present in the complex form. Further investigation of cell growth on humic acid in an UPW system may involve exposing the substrate to UV light, which will break down the structure and may make it more available to the bacteria. The less complex form is more likely to be present in an actual UPW system.

Both strains of *R. pickettii* grew to a high cell density on cellobiose, though strain MF254A showed an appreciable lag phase before growth commenced. Since *R. pickettii* 3A1 can degrade this compound, it implies that cellulose acetate membranes, occasionally still found in reverse osmosis systems, may be a source of nutrients for these cells. This may further explain the occurrence of *R. pickettii* within the polishing loop of UPW systems.

Many UPW systems purge the storage tank with nitrogen to remove oxygen (to minimize bacterial contamination) and carbon dioxide (to prevent ionic loading of the mixed-bed ion exchange resins). With respect to the *nifH* genes previously identified in *Bradyrhizobium* sp. 5F3 [23], we investigated whether this isolate could grow with sodium succinate as sole carbon source and fix nitrogen from the gaseous nitrogen blanket added. The results suggest that *Bradyrhizobium* sp. may be capable of nitrogen fixation within UPW systems, though clearly, this warrants further investigation. If nitrogen blanketing is confirmed to support growth of *Bradyrhizobium* sp. in UPW, argon offers promise as a replacement gas for use in UPW storage tanks.

Each bacterial strain tested in this study was capable of rapid proliferation in UPW to a level that was maintained for an extended period of time. In this case, the cell number was maintained for up to 6 months. Similar results have been reported previously for *Pseudomonas* sp. inoculated into distilled water. For example, Favero *et al* [14] described the behavior of *Pseudomonas aeruginosa* in mist therapy unit reservoir water and found that the organism increased its cell concentration from 100 to 1×10^7 CFU/ml within 48 h. This level was sustained for the remainder of the experiment (42 days).

Further work by this group showed similar growth trends with strains of *P. cepacia* and *P. aeruginosa* in both commercially prepared sterile distilled water [7], and water from a prototype distillation still, in which product water was continuously recycled to the still for redistillation [21]. In most cases, the final cell concentration attained did not decrease for up to 12 months. Interestingly, after stabilizing the cell numbers, many bacteria apparently became resistant to antimicrobial agents, such as chlorine dioxide, quaternary ammonium compounds, alkaline glutaraldehyde and acetic acid [14,15].

Other bacterial genera have also been reported to survive for extended periods in distilled water, including *Mycobacterium* sp. [8], *Yersinia* sp. [19] and *Bradyrhizobium* sp. [29,36]. It is noteworthy that in our study, *Bradyrhizobium* sp. 5F3 did not proliferate at an initial cell concentration of 10^7 CFU/ml or above. This correlates well with the observations of Ozawa and Doi [29], suggesting that there is an upper threshold concentration of cells that can be sustained in water.

It is important to emphasize that in our study, the bacteria were inoculated into UPW, not distilled water. It has been suggested previously that the cell increase in distilled water is due to the microorganisms growing on organic compounds dissolved in the water [7,8,14,29]. According to the ASTM, levels of organics in distilled water should be in the range of 50–200 ppb, depending on the method of preparation [4]. However, the TOC of UPW should ideally be less than 1 ppb, with a final resistivity of 18.2 MΩ cm. Therefore, if dissolved organics are the main carbon and energy sources for the bacteria, we would expect significantly lower cell yields in UPW than distilled water. Clearly, this was not the case.

We, therefore, propose that the observed increase of bacterial cell numbers (and total protein) in UPW may be due to a combination of the following strategies: (i) the bacteria were utilizing internal storage polymers such as poly-β-hydroxybutyrate, polyglucose or polyphosphate [31], or could degrade components of their EPS; (ii) the TOC in the UPW (0.5–2.0

ppb) was easily assimilable and/or the bacteria had higher affinity uptake systems for the available carbon sources. Increased uptake systems and low maintenance energies have previously been reported as likely characteristics of oligotrophs [31,38]; or (iii) cryptic growth was an influencing factor, yielding carbon and energy sources that the bacteria readily utilized.

Current standards for the production of UPW state that bacterial contamination should not exceed 1–10 CFU [3]. However, these standards make no reference to the numbers of dead cells present in the water. As we have previously reported, dead cells contribute to a significant percentage of the total number of bacterial cells present in UPW [26]. After demonstrating the occurrence of cryptic growth in *P. szygii*, there is clearly a need to address this issue.

Other workers have also shown the influence of cryptic growth in starving populations of bacteria. Using a strain of *P. fluorescens*, Dorofeev and Panikov [13] demonstrated that after 32 days of starvation, 90.5% of the biomass consisted of cells that had grown cryptically. Similarly, Banks and Bryers [5] found that *P. putida* could utilize 30–60% of the soluble organic material released following the sonication of bacterial cells.

Our data suggest that *R. pickettii* MF254A could grow readily at the expense of heat-killed cells. This significant increase was observed only with heat-killed, and not UV254-killed, cells. During heat treatment of the cell suspension, the high temperatures probably converted the polymeric particulates into simple monomers that were more easily degraded by the bacteria. Since many industries maintain their UPW in the polishing loop at 80°C or higher, the elevated temperature may actually contribute to biocontamination within the system.

Mono-species biofilms are rare in nature, so it should also be considered that even if large-molecular-weight compounds are present, co-metabolism by two or more species may occur. Furthermore, since most UPW systems contain UV185 lamps within the polishing loop, any large compounds are likely to be degraded to lower-molecular-weight carbon compounds.

It has been suggested that cryptic growth is probably significant in growing microbial cultures that can utilize a broad substrate range [25]. With respect to the relatively broad substrate range shown by *R. pickettii* MF254A and the rapid growth on heat-killed cells, cryptic growth seems a likely survival strategy by this organism in UPW. In contrast, our data suggest that cryptic growth in *R. pickettii* 3A1 is probably not a significant means of obtaining carbon for this microorganism. Since this strain appears less oligotrophic than MF254A, it may simply have not adapted sufficiently to scavenge all traces of carbon.

Following cell lysis, amino acids, carbohydrates and RNA are believed to be the main components released into the surrounding environment [28]. The ability of *R. pickettii* MF254A to grow rapidly on most amino acids and carbohydrates tested in this work may explain its pronounced ability to grow cryptically in UPW. Conversely, the very low cell yield observed when *Bradyrhizobium* sp. grew on the dead cells might reflect its narrower substrate range.

Cryptic growth did not occur in any of the bacterial strains tested when the cells were killed by UV254, which kills bacterial cells by inhibiting their ability to metabolize and reproduce. Although UV254-killed cells would ultimately lyse in an UPW environment, it seems possible that the exposure time of 20 min was insufficient to achieve this. In industrial UPW systems, the product water is continually recycled to the storage tank and, hence, is repeatedly subject to both UV254 and UV185 irradiation. UV185 light causes the formation of free radicals in UPW and these radicals may lyse bacterial cells. Therefore, even though we did not observe cryptic growth in our laboratory experiments when cells were killed using UV254 light, in an actual UPW system, UV-killed cells may be a source of nutrients to support cryptic growth. Any bacteria in the polishing loop may have a weakened cell membrane structure after exposure to UV185 light and thus serve as a source of nutrients for other cells in the system.

In conclusion, our results suggest that bacteria can adapt to an oligotrophic existence and may gain the ability to scavenge carbon sources as required. Whilst all of the bacterial strains tested could survive for prolonged periods in UPW, the final cell yields appear to be dependent on the strain and initial number of cells present. Finally, we propose that cryptic growth may be a significant source of carbon and energy to the contaminating bacteria within UPW systems.

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