Characterizing the Microbiota of a Pharmaceutical Water System-A Metadata Study

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Abstract

Bacterial populations inhabiting pharmaceutical grade water systems were investigated over a fifteen year period. The systems analyzed were mains water, purified and Water-for-Injection (WFI). Samples of water were tested by membrane filtration and the samples cultured using R2A agar. Culture based methods and phenotypic identification methods were used to characterize the isolates. The research was undertaken to produce an in-depth study of the microbiota of pharmaceutical grade water systems. The results presented act as a benchmark for industrial and pharmaceutical microbiologists to review comparable systems against, as well as to present a review of the typical culturable microorganisms recoverable from pharmaceutical water systems.

Keywords: Water; Water Systems; Microflora; Microbiota; Pseudomonads; Water-For-Injection; Purified Water; Pharmaceutical; Manufacturing; Bacteria

Introduction

Water is a key part of the pharmaceutical industry. Water is used for cleaning; as an ingredient for aqueous sterile and non-sterile products; for hand washing; and as the steam supply to autoclaves, among other uses. Due to its criticality in pharmaceutical production, microbiological control of water is of great importance. Because water is ever present, each grade of pharmaceutical water is a potential source of microbiological contamination, especially when not properly controlled [1]. Control is not only about numbers of microorganisms recovered through bioburden testing [2] for microbiologists additionally need to understand the types of organisms present within water. This is in order to look for changes to trends and to understand if they are indicators of more serious problems (like biofilms) or if they present a special risk to products (and thus to patients). This paper reviews the types of bacteria found within the microcosms of pharmaceutical water systems.

Even though most pharmaceutical water systems are controlled, microorganisms will sometimes be present, albeit in low numbers. Such recovery will be uneven (microbial distribution in water follows Poisson distribution, in that bacterial count do not fall into homogeneous groups). The types of aquatic bacteria recovered from pharmaceutical grade water systems will be either autochthonous and allochthonous.

The need for microbiologists to consider the constant and changing patterns of microorganisms found in water systems are varied. These reasons include the need [3]:

a) To understand the most commonly occurring isolates which make up the micro flora in the different water systems and to see if there is any relationship between different grades of water.

b) To build up such a picture so that, data can be compared and trended over subsequent years.

c) To examine the type of microorganisms to see if the types are similar to those expected to be found in a water system.

d) To take from the most commonly occurring isolates microorganisms to become part of a microbiological culture collection for future reference.

e) To use the most commonly occurring microorganisms for media growth promotion testing (the periodic challenging of culture media by so-termed environmental isolates is an expectation of regulatory authorities under the auspices of Good Manufacturing Practice).

f) To have the information in a format for trending, so that microbiologists and quality personnel can react to any changes to trends.

Some or all of the above may be applicable, depending upon the pharmaceutical facility of the different reasons outlined, establishing a benchmark is important. This is not a task that, hitherto this paper, has been aided by literature. There are few published references as to the expected micro flora in processed water and even fewer that pertain to the pharmaceutical industry. The paucity of studies is the reason for developing this review: to enable microbiologists to compare and to contrast these findings when establishing their own similar reviews. As general guidance, water systems should be characterized at least annually, with some reference made to the typical and expected microorganisms every time an action level result is recorded from monitoring.

In relation to the available studies, a review by Mcalister et al. detailed the typical microorganisms recovered from a purified water system as *Ralstonia pickettii* and *Bradyrhizobium* spp*.* The former seems probable based on the author's own observations; however the latter would seem more expected from freshwater habitats. Mcalister and colleagues noted that both bacteria identified have adapted to an oligotrophic existence, with *R. Pickettii* notably adapting and surviving to the low-nutrient conditions particularly well.

An alternative study of a purified water system indicated that the species of *Pseudomonas*, *Flavobacterium* and *Acinetobacter* were particularly ubiquitous. The proportions occurring frequently were [4]:

Pseudomonas aeruginosa 32.05%

Pseudomonas pickettii (*Ralstonia pickettii*) 23.08%

Pseudomonas vesicularis 12.82%

Pseudomonas diminuta 11.54%,

Flavobacterium aureum 6.42%

Pseudomonas fluorescens 5.13%

Acinetobacter lwoffii 2.56%

Pseudomonas putida 2.56%

Pseudomonas alcaligenes 1.28%

Pseudomonas paucimobilis 1.28%

Flavobacterium multivorum 1.28%

The percentages above were recoveries from a total of 78 isolates.

A further study, by Kulakov *et al.* [5] identified the following bacteria from a pharmaceutical system (listed in order of their occurrence):

- *Ralstonia pickettii*

- *Pseudomonas fluorescens*

- *Bradyrhizobium* spp*.*

- *Pseudomonas saccharophila*

- *Sphingomonas* spp*.*

- *Flavobacterium* spp*.*

- *Burkholderia* spp*.*

- *Stenotrophomonas* spp*.*

In addition there are other references as to the expected microflora in fresh and mains water and it can be assumed, to some extent, that several of the microorganisms noted will be found in pharmaceutical water generation plants, originating via the supplied potable water. Among the biota of freshwater, these organisms are:

- Mainly Gram-negative bacteria. There is a high level of taxonomic biodiversity. Common species include: *Pseudomonas*

aeruginosa, Bdellovibrio bacteriovorus, Methylomonas methanica, Azobacter chrococcum and *Caulobacter vibrioides.*

- Very few Gram-positive bacteria are isolated; those that are identified are typically *Bacillus* spp.

- Bacteria are not the only microorganisms that inhabit source waters; there will be a complete ecosystem in operation which includes fungi, protozoans and algae.

It was also noted from such studies that the size of the bacteria vary with the supply of nutrients and other aspects of the microenvironment, such as pH, water flow, opportunities for surface attachment and so on [6]. With reference to ultrapure industrial water (prepared in a similar way to pharmaceutical grade water), studies suggest that the following microorganisms are common: *Ralstonia pickettii, Bradyrhizobium* sp., *Pseudomonas saccharophila,* and *Stenotrophomonas spp* [7]. Thus the literature would suggest that in purified water non-fermenting Gram-negative rods appear to predominate [8].

Across these reviews, for all types of water system, there is commonality with the genera *Pseudomonas* and *Ralstonia.* Significantly, the Gram-negative species predominate over Gram-positive ones.

Due to few studies being orientated to the pharmaceutical or healthcare setting, the review outlined in this paper was undertaken. The facility studied, a pharmaceutical organization located in the south-east of England, has the following types of water:

a)   Mains (or potable) Water;

b)   Purified Water;

c)   WFI (Water-for-Injection)

Each of these is of a different grade and the list descends with an increased expectation of microbial control (that is tighter limits apply to Water-for-Injection that for mains water) [9]. Mains water is supplied by a utility company and is of "drinking water" (potable water) quality. This water is supplied chlorinated, although a pharmaceutical plant would typically remove the chlorine and soften the water through demineralization.

Purified water is high grade water, produced by reverse osmosis. Reverse osmosis units use a semi-permeable membrane and a substantial pressure differential to drive the water through the membrane to achieve chemical, microbial and endotoxin quality reductions (the applied pressure is used to overcome the osmotic pressure in the water). Reverse osmosis occurs as the pressure is applied to the concentrated side of the membrane. This forces purified water into the dilute side. The rejected microorganisms from the concentrated side are then washed away.

WFI is produced by either distillation or by two-stage reverse osmosis. Distillation functions by turning water from a liquid to a vapor and then from vapor back to liquid. WFI is usually stored and distributed hot (at around 80°C) in order to meet microbial quality requirements. In the system studied in this paper, the WFI

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was produced by distillation. Produced WFI is held in a collection tank and then distributed around a pharmaceutical facility via a loop. The key aspect for the avoidance of contamination is the flow rate, and design of the pipes in terms of smooth finishes and avoidance of areas where the water can stagnate.

The results from the monitoring of water systems are assessed from heterotrophic microbial counts against pre-defined alert and action levels. Heterotrophs are organisms, including bacteria, yeasts and moulds that require an external source of organic carbon for growth.

For the microbiological examination of the water, the method of testing was membrane filtration through the use of cellulose acetate 0.45 μm filter. The filters were placed onto R2A agar and subjected to a temperature regime of 20-25°C for fourteen days for mains water and 30-35°c for five days for the purified water and WFI. The reason for these different regimes is that the European Pharmacopeia, for GMP facilities, requires the use of the stated temperature and time. For mains water, a pharmaceutical facility can select the cultural conditions. In this case, the conditions for mains water were considered to be optimal. For WFI, there is an additional requirement to screen the water for bacterial endotoxins (typically this is using the Limulus Amebocyte Lysate (LAL) test). This test falls outside the scope of this paper.

Selecting optimal cultural conditions relates to a classic dilemma in microbiology of concerning the most appropriate temperature, time and culture media to use. R2A was developed during 1980s by American bacteriologists Reasoner and Geldreich [10].The medium was formulated, with a low level nutrient, so that it would detect a higher proportion of heterotrophic bacteria. The theory was that as bacteria in water are subject to nutritive depleted conditions, then they would be more likely to grow on growth media prepared to more closely match those prevalent conditions.

Tests showed that R2A yielded higher counts when incubated for 5–7 days at 20°C or 28°C, when compared with more nutrient-rich growth media such as nutrient agar or tryptone soya agar. Moreover, the medium permits the examination of larger sample volumes using the membrane filtration method. Thus R2A, at the optimal incubation conditions probably records better counts but does not grow the full range of microorganisms present [11]. The reason for selecting and using R2A was based on its expected use by regulatory authorities (such as described in the European Pharmacopeia). The main variation from Reasoner and Geldreich's pioneering studies was that the pharmacopeia requires Water-for-Injection and purified water to be incubated at a higher temperature (30-35°C), whereas purified water and mains water is left to the discretion of the user. In the study described in this paper, 20-25°C was selected for mains water, given that these are cold water systems. Although Water-for-Injection circulates at > 85°C, a higher temperature was not selected for incubation on the basis that the recovery of organisms that would survive at such temperatures (so-termed extremophiles) was unlikely and on the risk-based assumption that the most common isolates would be in association with sink outlets and sampling valves - locales where the water is cooled to an ambient temperature of 20-30°C.

It is important to note that the microflora recovered are those that could be isolated using the culture media employed under defined incubation conditions. It is recognized that all culture media used is selective and most microbiological culture media is designed to select a wide population and that the downside of this is a limitation in detecting fastidious bacteria or those with specific physiochemical properties. Moreover, water systems will contain considerable higher numbers of bacteria that are culturable using standard methodologies. These are the so-termed "viable but non-culturable" organisms [12]. Several bacteria, mostly Gram-negative, have been shown to proceed into a viable but non-culturable stage when exposed to different environmental or nutritional stresses. For this reason, a method of assessing microbial numbers in a sample of water, such as flow cytometry, will indicate far greater numbers of microorganisms than any cultural method can show. This is because many of the microorganisms listed, particularly the prosthecate bacteria, the *Mycobacterium* species, spirochaetes, and *Thiobacillus* species are unable to grow on the heterotrophic plate count media; for some organisms it is too rich, and for others the cultural conditions are unsatisfactory.

A further limitation is that the methods for water sampling and testing bias detection towards those in the planktonic (free floating state) rather than those microorganisms in the benthic state (attached to a substratum) or part of a biofilm community.

Furthermore, the microorganisms found in pharmaceutical water systems are mainly stressed, slow-growing strains characterized by long incubation times before growth can be detected. This results in the identification of such micro-organisms being somewhat problematic. Identifications are improved through the use of an intermediate nutrient agar, like R3A, as a subculture medium [13].

Methodology

The samples collected for the analysis where taken from a fifteen year review period (2000 to 2014). During this time, some 54, 140 samples were collected and tested; and, from these, 1,151 bacteria were isolated and identified.

The test method involved aseptically collecting 100 and 200 ml samples of water in sterile containers. The containers for collecting the mains water contained a neutralizer to eliminate chlorine residues. Samples were either tested within two hours or they were stored at 2-8°C for up to 24 hours before testing [14]. The method of testing was membrane filtration and the agar used was R2A, according to the incubation conditions discussed above.

After incubation, where colony forming units could be discerned, samples were submitted for identification. Identification involved the subculture from R2A onto a more nutritious R3A medium [15]. For identification, phenotypic methods were used: either API test kits (biomérieux, Craponne, France), such as the API 20NE system, or an omnilog system (Biolog, Hayward, CA, USA). API kits produce color changes upon microbial growth to different chemical substrates [16]. The omnilog system is a standardized, rapid method for determining bacterial oxidation of different carbon sources

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simultaneously. The results of these reactions are compared to a database and a microbial identification is then provided [17]. With any identification system, the results obtained are affected by the comprehensiveness of the database and many systems are relatively weak for organisms found in an industrial environment compared with those recovered within the clinical setting. It also stands that over the time period of the study, a number of microorganisms will have been re-classified as advances in ribosomal DNA sequencing reveals new phylogenetic differences.

Results

Mains (potable) water

The incoming mains water to the pharmaceutical facility was chlorinated and samples from a nearby, off-site supply point are screened by a water utility company. The mains water samples were drawn from a holding tank within the pharmaceutical facility.

Over the period of review, 1,040 samples were taken. Samples typically recovered microorganisms although few samples (201) were above the action level of 30,000 Colony Forming Units (CFU)/100 ml. The action level was based on the recommended level for drinking water within the European Union. From the 201 samples above the action level, 504 isolates were identified. The relatively low number of samples above the action level was a reflection of the overall quality of the in-coming water. The relatively low microbial populations reflected the in-coming water being chlorinated.

The primary genera recovered were Gram-negative bacteria; however, a diversity of Gram-positive organisms was also found. The primary genera are displayed in Table 1 below.

In terms of the most numerically occurring species, these were (Table 2):

The relatively low number of species occurring more than once is partly a reflection of the low number of out-of-limits samples alongside the diversity of different species present in the water system. All of the most commonly occurring species were Gram-negative bacteria.

Purified water

The process of manufacturing the purified water was via reverse osmosis. Here deionized water was subjected to the filtration process.

For the study, 6,300 samples were tested. Of these, some 315 samples exceeded the action level of 100 CFU/100 ml (5%) and 347 isolates were recovered. The primary genera are displayed in Table 3.

The most common genera were "Pseudomonad type" organisms, with *Ralstonia* being the most prevalent (these were all species of *R. Picketti,* as shown in Table 4*)*.

R. Pickettii and *B. Cepacia* were overwhelmingly the most common isolates from the purified water system.

Table 1: Primary bacterial genera from mains water system.

Rank	Genus	2000 - 2014: Number detected (Percentage of total, n=504)
1	*Pseudomonas*	121 (24%)
2	*Brevundimonas*	70 (14%)
3	*Sphingomonas*	50 (9%)
	Bacillus	37 (7%)
4	*Moraxella*	34 (7%)
	Micrococcus	33 (7%)
5	*Ralstonia*	31 (6%)
6	*Stenotrophomonas*	22 (4%)
7	*Burkholderia*	18 (4%)
8	*Methylobacterium*	10 (2%)

Table 2: Primary bacterial species from mains water system.

Rank	Major species	2000 - 2014 – number detected
1	*Pseudomonas fluorescens*	33
2	*Brevundimonas vesicularis*	21
3	*Ralstonia pickettii*	16
4	*Pseudomonas stutzeri*	11
5	*Sphingomonas* sp.	9

Table 3: Primary bacterial genera from a purified water system.

Rank	Genus	2000 - 2014: Number detected (Percentage of total)
1	*Ralstonia*	105 (30%)
2	*Burkholderia*	80 (23%)
3	*Pseudomonas*	31 (9%)
4	*Moraxella*	23 (7%)
5	*Flavimonas*	20 (5%)
6	*Stenotrophomonas*	14 (4%)
7	*Ochrobactrum*	13 (4%)

Table 4: Primary bacterial species from a purified water system.

Rank	Major species	Number detected
1	*Ralstonia pickettii*	105
2	*Burkholderia cepacia*	80
3	*Moraxella spp.*	23
4	*Stenotrophomonas maltophilia*	13
5	*Ochrobactrum anthropi*	12
6	*Flavimonas oryzihabitans*	10

Water-for-Injections

Few microorganisms are typically recovered from Water-for-Injection (WFI) systems. This is due to the nature of the method of producing the water (either reverse osmosis or distillation of purified water) and the distribution of the water, where the

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water is typically held at 80°C or higher. Risk of contamination is further reduced if the water system is of a sanitary design, with pipework designed to avoid so-termed "deadlegs" (areas where the water flow is disrupted) and the water held in a state of continual movement.

For the sample time period 46,800 WFI samples were taken and tested. The primary genera (displayed in Table 5) were Pseudomonad and related genera. The review of data for the fifteen year time period shows that samples rarely exceed the specification for the water system (which is set by the pharmacopeia at 10 CFU/100 ml). Where microorganisms are recovered from test samples, the microbes isolated are invariably Gram-positive *cocci*. The recovery of these organisms will inevitably be a facet of testing or sampling.

Gram-negative bacteria are arguably the primary contaminants of WFI. From the 46,800 samples taken during the review period, only 300 samples detected Gram-negative rods (a rate of 0.6%) Of these 300 samples, 439 Gram-negative rods were recovered (less than two different organisms per sample.) The Gram-negative rods that are recovered fall into the genera set out in Table 5.

The table indicates that the most common genera are Pseudomonads and *Pseudomonad*-related genera. These organisms comprise 8 out of the 10 most frequently isolated genera. There was little variation with the recovery rates over the course of the review period. In terms of variation, 26 different genera were recovered, albeit most at very low levels.

In terms of species, where five or more different species could be discerned, the primary isolates are displayed in Table 6.

Table 6 indicates that the numerically most common species was *Ralstonia pickettii*, followed by *Burkholderia cepacia* and *Flavimonas oryzihabitans*. These numbers were consistent over the fifteen year review period.

Discussion

Understanding the total numbers of microorganisms within a given water system is important in order to compare those limits against guidance limits and for trending purposes. It is also important to perform regular microbial identification so that results can be compared against what is expected and so that contamination strategies can be devised against certain types of microorganisms [18].

With benchmarking and understanding the microbiota of pharmaceutical water systems, this paper has reviewed data collected over a fifteen year period. It is considered that those microorganisms put forward for identification were reasonably representative of those isolated. Whilst the representativeness and reliability of the data will be influenced by the geographical locale and methods of water purification, the long-term historical nature of the study and the similarities with other published literature provide a reasonable indicator and comparator for those wishing to carry out similar examination. Thus the species listed as the most commonly occurring in this paper

Table 5: Primary genera from a pharmaceutical WFI system.

Rank	Genus	Number detected (and percentage of the 439 gnrs)
1	*Pseudomonas*	35 (8%)
2	*Burkholderia*	28 (6%)
3	*Ralstonia*	27 (6%)
4	*Flavimonas*	21(5%)
5	*Moraxella*	17 (4%)
6	*Chryseobacterium*	13 (3%)
7	*Stenotrophomonas*	9 (2%)
7	*Brevundimonas*	9 (2%)
9	*Sphingomonas*	7 (1%)
10	*Ochrobactrum*	6 (< 1%)
11	*Pasteurella*	5 (< 1%)
12	*Ochrobactrum*	4 (1%)
12	*Bordetella*	4 (< 1%)
14	*Alcaligenes*	4 (< 1%)
15	*Serratia*	3 (< 1%)
15	*Aeromonas*	2 (< 1%)
15	*Chryseomonas*	2 (< 1%)
15	*Myroides spp*	2 (< 1%)
15	*Wautersia*	2 (< 1%)
20	*Comamonas*	1 (< 1%)
20	*Methylobacterium*	1 (< 1%)
20	*Acinetobacter*	1 (< 1%)
20	*Pantoea*	1 (< 1%)
20	*Achromobacter*	1 (< 1%)
20	*Cupriavidus*	1 (< 1%)
20	*Gordonia*	1 (< 1%)
20	*Microbacterium*	1 (< 1%)

Table 6: Primary bacterial species from a pharmaceutical WFI system.

Rank	Major species	2000 - 2014 – number detected
1	*Ralstonia pickettii*	49
2	*Burkholderia cepacia*	34
3	*Flavimonas oryzihabitans*	29
4	*Moraxella spp.*	16
5	*Pseudomonas fluorescens*	13
6	*Chryseobacterium indologenes*	12
7	*Stenotrophomonas maltophilia*	11
8	*Brevundimonas vesicularis*	5

can be benchmarked by other microbiologists responsible for overseeing their own water systems.

The key usefulness of such information is in tracking contamination to see if the pattern alters over time. Changes

to the microorganisms ordinarily recovered could signal a breakdown in control. Thus trending is an important part of the microbiological role. Another use for the data can be with selecting the most important microorganisms to be use for growth promotion testing for the release of culture media (challenging media with so-called "wild type organisms" for quality control purposes is a growing expectation by regulatory agencies.)

In terms of similarities and differences with pharmaceutical plants in other parts of the world, the diversity of mains water will be the most variable. This variation will depend primarily on two factors, the first of which is the catchment area. These will either be nutrient-poor (oligotrophic) upland rivers where the microbial count, even using direct counting methods, will seldom exceed a few thousand CFU/ml; to they will be nutrient rich (eutrophic) regions of lowland rivers, where the counts may well exceed one million per ml. The second factor is the season; the levels of microorganisms in natural waters follow a seasonal distribution curve controlled by the amount of available nutrients and temperature.

With the system studied in this paper, the range appears to be towards the oligotrophic end of the scale. Furthermore, across the fifteen years of the study, no significant seasonal variations were noted. The predominant isolate from mains water was *Pseudomonas fluorescens. P. fluorescens* can grow at ambient and lower temperatures [19]. The organism is more often associated with agriculture than water systems, suggesting run-off from fields into the water feeding into the reservoirs for the water collected by the providing utility company. Like other Pseudomonads, the bacterium is well-equipped to survive in aquatic environments. The organism is generally non-pathogenic.

The second most prevalent organism was *Brevundimonas vesicularis. B. vesicularis* was formerly classified as a Pseudomonad [20]. The organism is commonly associated with water and slime. It is non-pathogenic.

The third most common isolate was *Ralstonia pickettii. R. Pickettii* is common to soil and water [21]. Together with the other primary species from mains water, its occasional presence is unsurprising.

With the purified water system there was a close similarity with the WFI system, in that *R. Pickettii* and *B. Cepacia* were the most common isolates. These organisms seem to be indigenous to an oligotrophic purified water environment. Given the similarity of the bacteria recovered, the primary organisms are discussed in relation to the WFI system below.

With WFI, the most common isolate was *Ralstonia pickettii.* This organism, as noted above in relation to mains and purified water, appears common to water environments. Furthermore, the bacterium is an associated community member of biofilms in pipes [22] (along with other non-fermenting Gram-negative rods, particularly Pseudomonads)[23]. Given that the organism capable of surviving in areas with a very low concentration of nutrients, its recovery from the low-nutrient conditions of WFI is unsurprising.

As to whether this organism presents a particular risk to pharmaceutical process is debatable. Any high levels of Gram-negative organisms is problematic, especially in relation to endotoxin generation. It has been estimated that as few as 2.5 x 10^2 CFU/ml of *B. Cepacia* and 3.3 x 10^3 CFU of *P. Fluorescens* will give a detectable level of endotoxin with the LAL test (around 0.06 EU/ml) [24]. With *R. pickettii,* the primary risk to pharmaceutical processing is the bacterium's ability to pass through filters with small pore sizes, including the primary sterilizing grade filter (0.2 μm). If the organism is found in high numbers, then possibly consideration should be given to using a filter with a smaller pore size (such as 0.1 μm.) In hospital settings the organism could pose a risk to the immunocompromised host [25]. In a clinical setting, as with a hospital water system, the bacterium is a nosocomial pathogen associated with those who are debilitated or immunosuppressed.

The second most populous isolate from WFI was *Burkholderia cepacia*, or, more precisely, members of the *Burkholderia cepacia* complex (BCC), of which there are 18 different species [26]. These aerobic organisms are found in soil and water and can also survive for long periods in low-nutrient moist environments, which again makes them probable survivors within pharmaceutical grade water systems [27]. *B. Cepacia* is a human pathogen and can cause pneumonia in immunocompromised individuals.

The third most common isolated with WFI is *Flavimonas oryzihabitans*, which was formerly classified as a Pseudomonad. The bacterium is closely related to *Pseudomonas putida*. The bacterium is associated with water and biofilms, and it poses a potential risk to patients within the hospital environment as a nosocomial pathogen [28].

Across all three systems, *R. pickettii* is the most prevalent bacterium, and Pseudomonads (and related genera) predominate. Whether the recovery of these organisms reflects those bacteria that might survive most readily in the relatively harsh, low-nutrient environment of a pharmaceutical grade water system; or, alternatively, whether these recoveries reflect the limitations of the identification methods deployed is uncertain. Nonetheless, the bacteria recovered have adapted to survive in conditions were nutrients are scarce and where temperatures vary considerably. Pseudomonads are particular adept for surviving in such oligotrophic conditions; here, the water does not contain more than 1-5 mg/l of organic carbon. Survival of oligotrophic organisms in water systems is more likely to be through surface attachment in communities than in a 'free-floating' state.

In terms of organisms that were not detected across the period of review, one part of the microbial monitoring of pharmaceutical water systems often conducted in conjunction with total count determination is the screening for objectionable or indicator organisms [29]. With the system studied, additional screening was performed, using selective agars, for *Escherichia coli* and *Pseudomonas aeruginosa*. That these organisms were not discovered shows that microorganisms of faecal origin (coliforms) or particular opportunistic pathogens (*P. Aeruginosa*) were unlikely to have been present in the source water. This was

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piece of null data was expected, given the theoretical quality of the incoming water, and satisfactory.

In terms of further study, a future strand of work would be to determine how well the different isolated flora, from a given system, survive in other water systems. How would organisms found from a purified water system fare in a WFI system, for example? Another area to explore further is the precise inter-relationship of microbial content across the different systems and whether microbial transfer occurs. The use of genotypic microbial identification methods, such as ribotyping, could assist with this type of inquiry. It is noted, for example, that the ubiquitous *R. Picketti* is similar to another of the isolated genera - *R. Insidiosa* - and true differentiation is only possible at the genetic level [30].

Conclusion

This paper, in reviewing data from three inter-connected pharmaceutical water systems (mains, purified and Water-for-Injections), has established a benchmark of the typical microorganisms that can be isolated and recovered on culture media. Whist is recognized that each facility and geographical locale will differ; the types of organisms recovered bear some similarity to earlier reviews of industrial and laboratory water. Therefore, the results compiled provide empirical support for some of the more theoretical discussions about the microbial ecology of pharmaceutical grade water. The study revealed that the most common isolates are Pseudomonads and related genera like *Ralstonia*. Tracking and trending water microbiota is an important part of pharmaceutical microbiology, providing the basis for evaluating microbiological risks to products and environments, and this paper, through its long-term historical review, is designed to help with this review process.

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