Hyperthermophilic Microorganisms and USP Hot Water Systems

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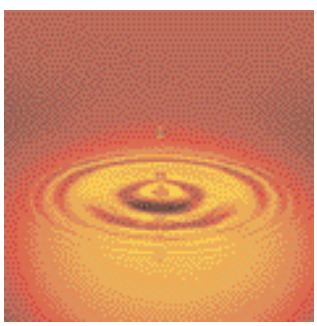

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Some industry practices not only increase costs without providing any real benefit, but they are based on misconceptions that have no scientific basis according to current research. Two such paradigms are that thermophilic and hyperthermophilic bacteria can contaminate hot water for injection (WFI) and purified water (PW) systems and that hot WFI and PW systems must be kept at temperatures ⩾70 °C.

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By definition, thermophiles love heat. In fact, thermophiles thrive at temperatures that will kill most other forms of life. Regular thermophiles (referred to as *thermophiles* in this article) are organisms that live at temperatures >45 °C and have an upper grow temperature limit of ~70 °C. Hyperthermophiles live at temperatures >70 °C and can have an amazing upper grow temperature limit as high as 113 °C (1). (For comparison, pure water boils at 100 °C at sea level.)

Within the framework of biology and physical biochemistry, the definition of *life* refers to cellular organisms whose characteristics are as follows: the capacity for metabolism (energy transformation), growth, response to stimuli, and reproduction (2). This definition is important because although some microorganisms can withstand high temperatures for a period of time, they cannot live at those temperatures because they are unable to grow and reproduce. In addition, one can define *high temperature* as the upper temperature range in which mesophilic organisms do not survive. Specifically adapted *hyperthermophilic* organisms grow and multiply at high temperatures—not simply tolerating this environment, but requiring it as their standard physiological condition (2).

The existence of thermophiles has been known for a long time, but hyperthermophiles were discovered for the first time about 40 years ago. Some biologists believe that hyperthermophiles may have been the first life to arise on Earth (3,4). Hyperthermophiles found in hot springs and deep-sea vents are the most studied of the extremophile types (5). However, they also can be isolated from compost, near volcanic vents and volcanic landscapes on land.

As Table I shows, only prokaryotes can live at temperatures ⩾65 °C. According to current literature, no plant or animal can live at temperatures >50 °C. The upper-temperature limit for eukaryotes is not much higher, only 62 °C.

In pharmaceutical applications, purified water (PW) and water for injection (WFI) are kept at temperatures between 70 and 90 °C to prevent the growth of microorganisms. Taking into account this temperature range, one may wonder about the possibility of thermophiles or hyperthermophiles contaminating these water systems. This is not a hypothetical question. In fact, in some cases, inspectors have questioned whether phar-

Table I: Upper temperature limits for growth of major living groups.

Living group	Approximate upper-temperature limit (°C)
Plants	50
Metazoa	50
Aerobic eukaryotes	62
Prokaryotes:	
Cyanobacteria	73
Methanogens	110
Mesophiles	45
Thermophiles	70
Hyperthermophiles	113

Adapted from References 51 and 52.

Carbon and energy nomenclature

- autotrophs: organisms that use CO_2 as a sole source of carbon for growth
- chemoheterotrophs: organisms that use (oxidize) an organic form of carbon to produce energy
- chemolithoautotrophs: inorganic redox reactions serve as energy sources (chemolithotrophic), and CO_2 is the only carbon source required to manufacture organic cell material
- heterotrophs: organisms that use organic carbon as a source of carbon for growth
- lithotrophs: organisms that oxidize inorganic compounds to produce energy
- phototrophs: organisms that use light as a source of energy.

maceutical companies monitor their WFI for the presence of thermophilic bacteria. They have stated that *Bacillus stearothermophilus* (from biological indicators) or other thermophilic bacteria may contaminate hot WFI or PW systems, which, at first glance, may seem possible. However, upon careful evaluation, it is deemed that this occurrence is extremely unlikely. Nevertheless, this misconception has become another paradigm in the pharmaceutical industry. Another paradigm that has deep roots in the pharmaceutical industry, especially in the parenteral and aseptic processing areas, is that hot WFI and PW systems must be kept at a temperature ≥70 °C to prevent microbial contamination. This article discusses these two misconceptions and the rationale for breaking these paradigms.

Characteristics of USP PW and WFI

United States Pharmacopoeia (USP) PW is produced with the use of several methods, including reverse osmosis, electrodialisis reverse, and continuous deionization. USP PW is stored and distributed either hot or at room temperature (RT). WFI usually is produced from the distillation of PW and is stored and circulated through the distribution system at 70–90 °C. USP requirements for PW and WFI are the same, except for microbiological considerations.

Both types of USP water must have a total organic carbon (TOC) content of <500 ppb (6). Usually, the TOC concentration is <200 ppb and in well-kept systems is <50 ppb. These latter values are significantly different from the TOC concentration normally found in potable water, which is >1 ppm. The

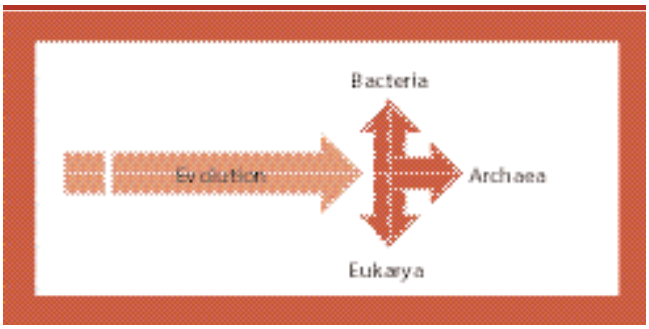

Figure 1: Three major domains.

dissolved organic carbon in seawater is rarely >1 ppm and is considered a low-nutrient environment (7,8). Particulate organic carbon usually accounts to 10–20% of the TOC. A large fraction of the latter is unavailable for microbial attack (6,7). Hence, one can deem that USP PW and WFI, as intended, constitute low-nutrient environments.

USP 26 Chapter 645 specifies that the conductivity must be <1.3 mS/cm at 25 °C (9). Again, both types of USP water easily meet the specification. Normal readings are usually <0.5 mS/cm at 25 °C. Although pH is no longer a USP requirement, typical pH values range from 5.0 to 7.0.

Thermophiles and hyperthermophiles that can grow in temperatures ≥65 °C

Table II lists a selection of microorganisms that can grow at temperatures ≥65 °C, according to the literature. Hyperthermophiles are all prokaryotes and mainly from the Archaea domain (see Figure 1). Archaean hyperthermophiles are so well adapted to high temperatures that they do not grow well at temperatures <60 °C, and some do not grow at all at temperatures <80 °C (10). Aerobic species are plentiful among the bacteria yet are rare among Archaea. Thermophiles are so numerous among Archaea that they are considered the trademark of the group. On the other hand, the majority of bacteria are mesophilic (11). Three major bacterial genera have microorganisms capable of growing at temperatures >65 °C: *Bacillus* (12,13), *Thermus* (14–17), and *Clostridium* (18–27). Uncommon genera, such as *Thermoleophilum,* have been documented, but they are found in very unique habitats. Because the Archaea is the less well known of the domains that contain these microorganisms, a more-detailed focus on its major characteristics is provided.

The Archaea

The Archaea are a very interesting group of microorganisms. They look and behave like bacteria and have a prokaryotic structure. However, although archaeans resemble bacteria and have some genes that are similar to bacterial genes, they also contain other genes that are more like the ones in eukaryotes. The following are unique biochemical and genetic features:

- Cell wall lacks peptidoglycan (no muramic acid or d-amino acids). Instead, they have pseudopeptidoglycan, polysaccharide, glycoprotein, or protein.
- Some have sterols in the cell membrane, which is a feature of eukaryotes.
- The structure of the cytoplasmic membrane is similar to that

Table II: Selected thermophilic and hyperthermophilic genera of microorganisms.*

Genus	Primary characteristics	Temp. range (°C)	pH	Respiration	Marine/ terrestrial	Habitats	Has potential to grow in hot WFI/PW?	References
Bacillus (bacteria)	All are endospore-formers; includes thermophilic, acidophilic and alkalophilic; freshwater and halophilic; autotrophs and organotrophs.	15–70	Wide	Aerobic and facultatively anaerobic	M/T	Ubiquitous	Yes	11,12
Clostridium (bacteria)	Spore-forming bacilli; includes psychrophilic, mesophilic, and thermophilic species; cannot tolerate a $pO_2 > 3\%$; organotrophs; require amino acids and growth factors.	⩽70	Wide	Strictly anaerobic to aerotolerant	M/T	Soil, hot springs, compost, normal intestinal flora of humans and animals.	No	17–26
Thermus (bacteria)	Obligate heterotrophs and grow on small amounts of organic materials; thermo and hyperthermophilic	37–80	2–10	Anaerobic can tolerate low oxygen levels	M/T	Thermal springs and hot water systems, different types of wastes (garden and kitchen wastes, sewage sludge) and in industrial composting systems	No	13–16
Thermoplasma (Archaea)	No cell wall (resemble mycoplasmas), acidophilic, thermophilic, organotrophs	33–67	1–2	Facultatively aerobic	M/T	Coal refuse piles that are heated and made acidic by other lithotrophic bacteria that oxidize iron pyrite (FeS) to sulfuric acid, marine hydrothermal systems	No	27, 28

*Table II continued on page 56.

found in bacteria and eukarya, but Archaea use ether linkages in lipids rather than ester linkages (28).

For the purpose of this article, the Archaea are categorized into the following five major groups:

- Hyperthermophiles: Gram-negative obligate anaerobes that require temperatures >50 °C for growth and can grow at temperatures >70 °C. They use sulfur as an electron acceptor (except *Sulfolobus,* which uses oxygen or ferric iron). The usual habitats are geothermally heated acidic soils or waters that contain sulfur (solfatara), such as those in Yellowstone National Park and near marine hydrothermal vents (29–33).
- Halophiles: Gram-negative organotrophic aerobes. Many have yellow-to-red carotenoid pigments that protect them against sunlight. They require a very high salt concentration to grow (as much as 10%) and require complex nutrients (vitamins, aminoacids, etc.) for growth (fastidious). Their habitats are hypersaline aquatic environments with alkaline pH such as Great Salt Lake, the Dead Sea, solar salt evaporating ponds (salterns), and salted fish (34).
- Methanogens (methane-producers): Gram-negative or positive, strictly anaerobic, fastidious (require vitamins, amino acids, and trace minerals). They gain energy by oxidizing hydrogen. For this reason, they use CO_2 as the oxidant, reducing it to methane (CH_4) in the process. The methanogens use simple organic acids such as acetate for synthesis of their cellular components. These organic acids are produced by other anaerobic bacteria as the end products of growth on cellulose and other polymers. The methanogens are abundant wherever organic matter is present in anaerobic conditions such as landfill sites, the rumen of cows, compost, and so forth (35–36).
- Sulfate-reducing species: hyperthermophilic, methanogenic anaerobes that extract electrons from hydrogen, lactate, glucose, and so forth and reduce sulfate, sulfite, or reduce thiosulfate to sulfide. Common habitats are high-temperature, sulfate-containing environments such as marine hydrothermal vents (37,38).
- Wall-less Archaea (*Thermoplasma*): acidophilic thermophilic aerobic organotrophs that lack a cell wall (resemble My-

Table II (continued): Selected thermophilic and hyperthermophilic genera of microorganisms.

Genus	Primary characteristics	Temp. range (°C)	pH	Respiration	Marine/ terrestrial	Habitats	Has potential to grow in hot WFI/PW?	References
Thermo-proteus (Archaea)	Hyperthermophilic, lithoautotrophic, organotrophic; uses hydrogen as energy and uses sulfur as electron acceptor	74–102	2–7	Obligate anaerobes	T	Geothermally heated, acidic soils or waters that contain sulfur and near marine hydro-thermal vents (marine solfataric situations)	No	29–31
Thermo-coccus (Archaea)	Hyperthrermophilic, organotrophic, uses sulfur as electron acceptor	70–104	4–7	Anaerobic	M	Same as thermoproteus	No	32,33
Pyrococcus (Archaea)	Same as thermococcus	70–105	6–8	Anaerobic	M	Same as thermoproteus	No	42, 43
Staphylo-thermus (Archaea)	Same as thermococcus	65–98	4–9	Anaerobic	M	Same as thermoproteus	No	44
Desulfuro-coccus (Archaea)	Same as thermococcus	70–95	4–7	Anaerobic	M	Same as thermoproteus	No	45
Pyrodictium (Archaea)	Hyperthermophilic, lithotrophic; oxidizes hydrogen for energy and uses sulfur as electron acceptor	80–110	5–7	Obligate anaerobes	M	Same as thermoproteus	No	47
Sulfolobus (Archaea)	Hyperthermophilic, organolithotrophic; aerobically oxidizes sulfur and uses oxygen or ferric iron as as electron acceptors	60–85	1–5	Aerobic	T	Continental solfatara fields, within hot, highly acidic water	No	48,49

Adapted and expanded from References 53 and 54.

coplasmas). They are found in coal refuse piles that are heated and made acidic by (other) lithotrophic bacteria that oxidize iron pyrite (FeS) to sulfuric acid and hydrothermal vents (39,40).

In general, most Archaea exhibit a chemolithoautotrophic mode of nutrition. That is, inorganic redox reactions serve as energy sources (chemolithotrophic), and CO_2 is the only carbon source required to manufacture organic cell material (41).

The energy-producing reactions in chemolithoautotrophic hyperthermophiles are anaerobic and aerobic types of respiration. Molecular hydrogen (H_2) serves as an important electron donor. In the natural environment, H_2 comes from volcanic gasses or as by-products of other microorganisms. Other electron donors are sulfide, sulfur, and ferrous iron. As is the case for mesophilic and aerobic microorganisms, some hyperthermophiles may use oxygen as an electron acceptor (41). However, oxygen-dependent hyperthermophiles are microaerophillic.

Anaerobic respiration depends on nitrates, ferric irons, sulfates, sulfurs, and CO_2. A large number of chemolithoautotrophic hyperthermophiles are facultative heterotrophs. These microorganisms can use organic material instead of inorganic nutrients whenever it is available (e.g., decaying cells). Heterotrophic hyperthermophiles produce energy either by aerobic or by various types of anaerobic respiration—using organic material as electron donors—or even by fermentation (10).

Achaean microorganisms are very exotic and singular. Their habitats and nutritional requirements are very exotic and singular as well. As a group, they require H_2, the simplest of all elements, to extremely complex growth factors. In addition, most archaeans are anaerobic or microaerophillic. Because of these characteristics it is extremely unlikely, if not impossible, for archaeans to grow in hot PW and WFI systems.

Genus Bacillus

It may be possible that bacterial spores could be present in the potable water supply or that spore formers might proliferate in the PW generation system and make their way into the distribution system. Because of their temperature resistance, spores could survive in the hot distribution loop and in extreme cases

Table III: Characteristics of *Bacillus* spp. capable of growing in the 55–65 °C temperature range.

Characteristic	*B. acidocaldarius*	*B. brevis*	*B. licheniformis*	*B. schlegelii*	*B. coagulans*	*B. stearothermophilus*
Growth at 55 °C	⩾90%	11–89%	⩾90%	⩾90%	⩾90%	⩾90%
Growth at 65 °C	⩾90%	⩽10%	⩽10%	⩾90%	⩽10%	⩾90%
Growth factors required	⩽10%	⩾ 90%	⩽10%	⩽10%	⩽10%	⩾90%
Autothrophic growth (CO/CO_2 and O_2)	⩽10%	⩽10%	⩽10%	⩾90% (H_2, CO_2, and O_2)	⩾90%	⩽10%
pH for growth	2–5	5–8	5–8	6–8.5	4–6	5.5–7
Special requirements	Requires NH_4^+ as a source of nitrogen	None	Requires NH_3 for growth in the absence of growth factors	Requires trace elements; heterotrophically needs acetate or succinate	Thermophilic acidophile	Sensitive to acid
Sources	Thermal, acid water and soil	Soil and foods	Soil	Lake sediments	Soil and acid foods	Soil, hot springs, desert sand, ocean sediments, food, and compost
Width of rod (μm)	0.9 –1.1	0.6–0.9	0.6–0.8	0.6–0.8	0.6–1.0	0.6–1
Length of rod (μm)	2– 3	1.5–4	1.5–3	2.5–5.8	2.5–5	2–3.5
Potential to grow in hot USP PW and WFI	None	None	None	None	None	Negligible

Tabulated and expanded from References 12 and 13.

might distill over with the stream in the distillation system. What would be the outcome of these scenarios? Table III lists three *Bacillus* species that can grow at temperatures ⩾65 °C: *B. stearothermophilus, B. brevis,* and *B. acidocaldarius.*

B. acidocaldarius grows in a pH range of 2–5 and requires NH_4^+ as a source of nitrogen. In addition, its normal habitats are geothermal vents and acid water and soil. Therefore, its nutritional requirements and acid habitats render *B. acidocaldarius* incapable of growing in hot PW and WFI systems (11–12).

B. schlegelii is another bacterium of this genus with the potential to grow in hot PW and WFI systems. However, it has less potential than *B. acidocaldarius* to do so. *B. schlegelii* can grow autotrophically in the presence of H_2, CO_2, and O_2 or heterotrophically given an appropriate source of carbon and energy such as acetate or succinate. *B. schlegelii* requires trace elements for growth such as zinc, sulfur, manganese, chlorine, boron, cobalt, copper, nickel, and molybdenum. Hot PW and WFI systems are the least-suitable places to fulfill these requirements (11,12).

The bacterial spores from *S. stearothermophilus* are extremely resistant to heat. For this reason, they are used as biological indicators in steam-sterilization processes. However, this bacterium is incapable of growing in hot PW and WFI systems because of its nutritional requirements. It has an absolute requirement for vitamins such as thiamine, biotin, and nicotinic acid. In addition, it needs several amino acids (e.g., arginine, histidine, and isoleucine) for growth. Simply stated, the nutritional requirements of *B. stearothermophilus* are well above the levels found in hot PW and WFI systems (11,12).

Bacillus spores can survive for long periods in hot PW and WFI but will not germinate because they require nutrients that are not present in these water systems. As time passes, these bacterial spores will die off.

Genus Clostridium

The genus *Clostridium* is very heterogeneous; however, all species are gram positive. It includes species that are moderately aerotolerant and others that are extremely fastidious to grow in the laboratory. Oxygen sensitivity restricts the habitat of the clostridia to anaerobic areas or areas with low-oxygen tensions. Growing and proliferating clostridia will not be found in air-saturated areas such as surface layers of lakes and rivers or on the soil surface. Therefore, clostridial cells will not grow in hot PW and WFI systems because the water is rich in oxygen. Moreover, the nutritional requirements of clostridial cells make it impossible for them to grow in hot PW and WFI. As in the case of *Bacillus* spores, *Clostridium* spores might be found in hot PW and WFI, but these spores will not germinate and eventually will die off (17–26).

Genus Thermus

The bacteria of the genus *Thermus* (Gram-negative) are obligate heterotrophs and grow on small amounts of organic materials present in water. They are usually isolated from biomass accumulations where they grow on the by-products of other microorganisms. This organism has been isolated from thermal springs, hot-water systems, and thermogenic composts. The optimum temperature for its growth is 70 °C, with a temperature range from 37 to 80 °C. The nutritional requirements

Table IV: Optimum and upper temperature limits for growth of some objectionable organisms and pathogens.

Bacterium	Optimum (°C)/Upper (°C)
Escherichia coli	37/45
Staphylococcus aureus	30–37/45
Pseudomonas maltophilia	35/41
Pseudomonas aeruginosa	37/42
Pseudomonas fluorescens	25–30/39
Vibrio cholerae	18–37/43
Listeria monocytogenes	30–37/45
Campylobacter jejuni	37–42/45
Bacillus cereus	28–42/55
Clostridium perfringens	37/50
Shigella spp.	35–37/47

Adapted and expanded from Reference 55.

of this non-spore-former bacillus make its proliferation in hot PW and WFI systems highly improbable (13–16).

A deep-rooted paradigm

According to common wisdom in the pharmaceutical industry, hot WFI and PW systems must be kept at a temperature ⩾70 °C to prevent microbial contamination. The origin of this paradigm stems from the food industry.

The question is, Why a temperature ⩾70 °C? This temperature value comes from FDA, where *CFR* 3-401 2001(last revision) FDA food code requires that food cooked for hot holding shall be cooked to a temperature of 60 °C (140 °F) and maintained at this or higher temperature. Food at a temperature of 60 °C or higher is considered to be safe from microbial contamination. However, the same document specifies to cook poultry to 82 °C (180 °F) and to cook beef to 71 °C (160 °F). Therefore, at one moment in time, someone recommended that to be safe it was best to keep WFI at temperatures ⩾70 °C. Although a valid scientific rationale was not presented, this became industry practice.

In food, the temperature "danger zone" is between 5 and 60 °C. It is understood that above 60 °C, food is safe from microbial contamination. However, the food pathogen with the highest growth temperature is *Bacillus cereus,* which can grow at temperatures as high as 55 °C. Therefore, there is a safety margin of 5 °C.

Most foods are good environments for the growth of microorganisms, but keeping foods at 60 °C protects them from microbial spoilage. Taking these facts into account and the fact that PW and WFI are very low-nutrient environments, one can deem that temperatures ⩾60 °C must provide a danger-free zone for USP hot PW and WFI systems.

Most objectionable microorganisms and human pathogens do not grow at temperatures >45 °C (see Table IV). Only *Bacil-*

lus cereus and *Clostridium perfringens* can grow at these temperatures. The upper-temperature limit of the former is 55 °C and for the latter is 50 °C.

The batch-pasteurization method has been used in the food industry for many years with excellent results. In this method, the food or beverage is exposed to a temperature of 60 °C for 30 min. Looking at Table IV, one can see that the data demonstrate that none of these microorganisms will survive for more than 60 min at 60 °C. After this pasteurization process, any human pathogen is inactivated. Only spores survive. The survival of spores in hot PW and WFI systems has no relevance because they cannot become vegetative and eventually will be inactivated or sequestered from the system by 0.22-μm on-line filters.

Discussion

Thermophiles and hyperthermophiles are adapted to unique environments, including pH, redox potential, salinity, temperature, and the composition and concentration of minerals and gasses. These alien conditions are necessities of their standard physiological requirements to live. Very few environments can simultaneously provide all of these requirements.

For microorganisms, in general, to be able to grow they need sources of energy, carbon, nitrogen, and trace minerals. The combination of low-nutrient contents, very limited availability of minerals, narrow pH range, and elevated temperature will limit the microorganisms with a potential to survive—let alone proliferate—in hot PW and WFI. Moreover, thermophiles from the genera *Bacillus, Clostridium,* and *Thermus* as well as Archaeans hyperthermophiles that withstand and can live at high temperatures have complex nutritional requirements that would be impossible for hot PW and WFI systems to provide.

Pseudomonas sps. and similar bacteria can grow in PW and WFI systems that are held at room temperature. These bacteria have ionophores that help them collect and store minerals. Also, low nutrient levels promote biofilm formation, which is another strategy to survive in low-nutrient environments. However, common pseudomonaceas cannot grow at temperatures >45 °C. Furthermore, pasteurization temperatures will kill all vegetative mesophilic bacteria within 30 min (50). Thermophiles and hyperthermophiles that can survive pasteurization will not proliferate and will die off as time passes.

Conclusion

Hot pharmaceutical water systems are extremely hostile environments for two major reasons: First, they are kept at high temperatures, near or above the temperatures used for the pasteurization processes. Second, as demonstrated previously, the organic materials, minerals, and salts are at very low concentrations. Therefore, it is extremely unlikely that thermophilic and hyperthermophilic microorganisms will live in hot PW and WFI systems. The likelihood is even lower for mesophilic microorganisms such as human pathogens and opportunistic pathogens. No objectionable microorganisms or human pathogens will grow at temperatures ⩾55 °C. In addition, as a bonus, there is no need to keep hot WFI and PW systems at a temperatures ⩾70 °C. Maintaining temperatures ⩾55 °C will guarantee the microbiological quality of these raw materials.

Recommendations

There is no need to screen or test hot WFI and PW systems for thermophilic and hyperthermophilic microorganisms. Set the storage and recirculation temperature of hot WFI and PW systems to 60–65 °C. The temperature range can be 55–65 °C. This approach will result in substantial energy savings.

Reduce the frequency of tests for total aerobic counts and coliforms because hot WFI and PW systems are self-sanitizing and self-preserving. There is no need to test for coliforms every day. It is recommended to sample the systems, on a daily basis, at the supply and the return of the distribution loop for TAC. Reduction of testing cost should be meaningful.

Any bacterial contamination found in hot WFI and PW should be a result of sample contamination, deficient GLPs, or poor maintenance of the system. Only *Bacillus* spp. should be isolated from these systems, and in well-kept systems this must be an unusual event. There is no need to validate hot WFI and PW systems for 21–30 days. Seven to 10 days should supply enough data to start up the systems. Revalidation of hot WFI and PW systems should be performed in 3–5 days in the case of minor changes and after shutdowns.

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