Validation Study and Quality Assurance of Pharmaceutical Water, Waterborne Microorganisms and Endotoxins

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Water for injection (WFI) and purified water are the most widely used and stringently regulated raw material in pharmaceutical manufacturing. WFI is utilized for a parenteral drug product. Water system is monitored at frequent and routine frequency for demonstrating the overall system control and stability of performance. The critical ports demonstrating systemic control should be monitored more frequently. For reducing the overall risk of microbial contamination or microbial build-up, it is important to develop appropriate alert and action levels. The assignment of alert and action levels should be performance-based, derived from the historic data and well below water specifications. These levels and overall excursion rates should be assessed annually. An action level should not be established at a level equivalent to the specification. Consecutive or multiple alert level excursions and each action level excursion should be comprehensively investigated with appropriate corrective and preventive action. It is important to analyze the efficacy of the corrective and preventive action to reduce the overall excursion rates.

Key words: Validation study / Quality assurance / Pharmaceutical water / Waterborne microorganisms / Endotoxins.

INTRODUCTION

Water is the most widely used raw material in the production, processing, and formulation of pharmaceutical and biotechnological products. Therefore, control of the manufacturing process, microbiological and chemical qualities of water throughout the production, storage and distribution processes is critical.

Regardless of the water types and grades, the quality of water is stringently regulated. Many different grades of water used in pharmaceutical manufacturing and various pharmaceutical purposes. Compendial documents describe their uses, method of preparation and quality attributes. There are two broad categories of waters: bulk waters and packaged water. Bulk waters are typically produced on site where they are used; whereas packaged waters are produced, packaged and sterilized to preserve microbial quality throughout their packaged shelf life.

This review summarizes the general requirements for monitoring the water systems for the waterborne microorganisms and endotoxins from gram negative microorganisms.

WATER TYPES

It is estimated that there are around 70 different types of bacteria in wastewater (Traeger, HL, 2003). Several different types of microbes cross water-treatment and are found in pharmaceutical waters. Most microbial contaminants are gram negative bacteria that pose the additional risk of endotoxin contamination of waters used for parenteral production.

Water used as an ingredient for pharmaceutical preparations must meet the requirements for purified water, water for injection, or one of the sterile forms of water covered by the pharmacopoeia. Pharmacopoeia is a useful guide because it contains not only information on water testing and minimum water quality standards but also information on the manufacture of the various types of pharmaceutical waters as well as microbial control and validation of water systems. In general, pharmaceutical waters can be divided into two groups: bulk water manu-
ufactured on-site and packaged water (produced, packed, and sterilized).

The bulk water is typically produced for use at the same site in large volume by a multiple-unit operation water system and employs a distribution system for use in the manufacturing purposes. These particular pharmaceutical waters must meet the quality attributes as specified in the compendial documents. Purified water and water for injection (WFI) are widely used in pharmaceutical manufacturing.

1. Purified and highly purified water: These waters are used as an excipient in the production of non-sterile products or non-parenteral preparations and in other pharmaceutical applications, such as cleaning of certain equipment and non-parenteral product-contact components unless otherwise specified.

2. Laboratory water: Purified water used, as applicable, for reagent preparation for the analytical tests and assays. The quality of the laboratory water to be used for preparing laboratory reagents should meet the needs of sophisticated analytical method and technologies with respect to conductivity and total organic carbon (TOC). In general the TOC conductivity levels should be very low.

Purified water must meet the requirements for ionic and organic chemical purity and microbial quality attributes as per United States Pharmacopeia (USP), European Pharmacopeia (EU) and Japanese Pharmacopeia (JP) or the other regulatory agencies such as World Health Organization (WHO) and US Environmental Protection Agency (EPA) which have standards for potable water.

The source water may be purified using unit operations that include deionization, distillation, ion exchange, reverse osmosis (RO), filtration, or other suitable purification procedures. These systems should be frequently sterilized to maintain microbial quality and microbiological monitoring to ensure water of appropriate microbiological quality is distributed at the points of use.

3. High purity water: European Medicines Agency (EMEA) has introduced a new grade of water called high purity water. It is defined as high quality water intended for use in the preparation of pharmaceutical products where a high quality of water is needed except where WFI is stipulated.

4. Water for Injection (WFI): WFI is used as an excipient in the production of parenteral and other preparations. In addition WFI is used for cleaning of certain equipment and parenteral product-contact components. WFI must meet all of the chemical, microbial and additional bacterial endotoxin specification. Since endotoxins are produced from Lipid A of gram negative bacteria (Figs 1, 2), the equipment and procedures used for the generation and distribution of WFI must be designed to minimize or prevent microbial contamination as well as remove incoming impurities and endotoxins from the starting water.

Differences in regulatory acceptance of production process for the feed water for the generation of WFI exist. Generation of WFI by RO is accepted outside of Europe and that of WFI through distillation process is accepted globally.

WATER SYSTEM DESCRIPTION

Production of pharmaceutical water employs sequential unit operations (processing steps) for reducing specific water quality attributes, protecting equipment and operation of subsequent treatment steps.

Pre-filtration
The purpose of pre-filtration operation is to remove solid particulate contaminants from the incoming source water supply of size ranging from 7 to 10 µm. These of removals prevent the damage to the downstream

FIG. 1. Cell wall composition of gram negative bacteria
Cited from Lucia Clontz (2009) Microbial Limit and Bioburden Tests, pp.13, CRC Press, NY.
system components from particulates that can affect the equipment performance. In general, the filtration technology uses primarily sieving effects for particle capture and a depth of filtration medium.

**Activated Carbon**

Activated carbon beds remove from the water certain low molecular weight organic material and oxidizing additives, such as chlorine and chloramine compounds, by adsorbing on to an activated carbon. This operation protects downstream stainless steel surfaces, resins, and membranes and maintains the chemical quality attributes. The activated carbon beds are highly susceptible to build up of microbial growth due to adsorption of or-
organic material and potential for hydraulic channeling.

**Softeners**

They utilize sodium-based cation-exchange resins and soften the water by removing water-hardness ions, such as calcium and magnesium. Water softeners can also be used to remove other lower affinity cations, such as the ammonium ion, that may be released from chloramidine disinfectants commonly used in drinking water. This unit operation protects the downstream processing equipment such as reverse osmosis membranes, deionization devices, and distillation units. The resin beds are highly susceptible for microbial biofilm formation.

**Deionization**

Deionization (DI), and continuous electro deionization (CEDI) remove the cations and anions. DI systems consist of charged resins that require periodic regeneration with an acid and a base. The anionic resin also removes some free endotoxin since endotoxins are negatively charged (Williams, KL. 2007). Like carbon beds, these resins are also highly susceptible to microbial colonization and proliferation.

**Reverse Osmosis (RO)**

Reverse osmosis (RO) units employ semipermeable membranes. The RO unit with the required controls purifies the water to improve chemical, microbial, and endotoxin quality and can accomplish a 1 to 2 log purification of most impurities and after passage through the membrane can generate purified water. The design and operation of RO units including membrane materials are extremely sensitive to sanitizing agents and to particulate, chemical, and microbial membrane fouling; RO units can be used alone or in combination with DI and CEDI units as well as ultrafiltration for operational and quality enhancements.

**Ultrafiltration**

Ultrafiltration (UF) is a technology most often employed in pharmaceutical water systems for removing endotoxins from a water stream. The UF devices work primarily by a molecular sieving principle. Ultrafilters with molecular weight cutoff ratings in the ranges of 10,000 to 20,000 Da are typically used in water systems for removing endotoxins (Williams, 2007). This technology may be suitable as an intermediate or final purification step. Like RO, successful performance is dependent upon pretreatment of the water by upstream unit operations. UF system is susceptible to water stagnation and could promote microorganism growth in back-up or standby units.

**Ultraviolet Light (UV)**

Typically, UV light is used in the post treatment destruction, removal and prevention of inoculation and proliferation of microorganisms is the key for reducing the microbial contamination risk. The low-pressure UV lights that emit a 254 nm wavelength are used for post treatment microbial control and destruction of ozone, when it is used for water sanitization purposes. However, a 189 nm wavelength is an effective barrier in preventing the microbial contamination risk. UV lamp must be sized properly for the required water flow rate. The disinfection performance of UV lamp depends on flow rate and water quality conditions. Although the UV light is commonly used, its effectiveness depends on contact time and depth. Generally the UV dose is computed using values of lamp intensity, residence time, distribution and water transmittance.

**Ozone**

Like UV, ozone is used in the post treatment destruction, removal and prevention of inoculation and proliferation of microorganisms. Ozone is a very strong oxidizing agent with powerful disinfecting properties and can be easily removed from water by exposure to UV light. It is an effective bactericidal, virucidal, fungicidal as well as sporicidal agent in water treatment systems. The efficacy of ozone to maintain the desired destruction of bacteria in the water system depends on residual dissolved concentration of ozone and contact time/sterilization time. It is suggested that a residual dissolved ozone concentration of at 0.3-1.0 mg/L for 2 hours is adequate. A frequent ozone sterilization at residual dissolved concentration of 4-5 mg/L and 15-20 minutes contact time are adequate to produce similar effect. (PDA, 2001)

**Final Filtration**

The final filter actually serves several purposes; major one purpose is to remove the particles that could enter the system as well as reduce bioburden. Two types of filters are used. They are 0.1 μm or 0.2 μm absolute filters. These filters do not perform sterilizing grade functions. (Meltzer, et al 2003)

**Distillation**

A distillation unit, usually referred to as a still, provides chemical and microbial purification via thermal vaporization, mist elimination, and water vapor condensation. It is noteworthy that the best distillation process cannot offer absolute removal of contaminating ions and endotoxin (Meltzer, et al 2004). Most stills are validated and able to remove at least a 3 to 4 log reduction of endotoxin and other nonvolatile organic impurities.
WATER SYSTEM MONITORING

Since the pharmaceutical waters are generally produced by continuous processes and used in products and manufacturing processes, it is extremely critical that water system operations, process controls, purity and quality of water should be validated to provide assurances of a controlled state, system stability and consistency. The monitoring schemes should provide early indications of system control and actions are taken to correct and prevent the risk loss of control. It is important to demonstrate the robustness of water purification process to consistently produce and maintained the chemical quality attribute of water. Therefore the nature and robustness of the purification process is directly related to the control of chemical purity. This paper also describes about the microbiological monitoring of water systems.

Water is the universal source for multiplication of variety of microorganisms. The control of microbiological quality of water is important because of its usage in variety of pharmaceutical manufacturing processes depending on the nature of the product. Some processes require stringent microbiological control while others do not. Failure to meet the microbial specifications requires robust investigations, product impact assessment and appropriate corrective and preventive actions. The investigational finding may have a serious impact on the acceptance of the pharmaceutical products.

The selection of the type of water used for manufacturing is based upon the type of product and process being used. The water quality should be evaluated for microbial bacterial endotoxin and chemical attributes. The testing verifies the adequacy of the process controls and quality control. The microbiological quality attribute is extremely critical for manufacturing of parenteral drugs and devices because it is used in product formulation and product contact component and equipment washing and final rinsing of equipment and devices.

Regardless of the nature of product, the control of microbial quality and chemical quality of water is important in relation to the safety of the product, and the control of microbial quality is critical to minimize the buildup and spread of microbial contaminant in the facility as well as manufacturing equipment.

In general, water systems are the most amenable for the use of automated monitoring technologies for monitoring chemical attributes, for example inline TOC and conductivity equipment have been used. Recently, significant advances have been reported for inline bacterial endotoxin and microbial monitoring. Such systems will revolutionize water testing and significantly increase the availability of real-time water quality data, improve efficiencies, productivity and decrease product release times.

Regardless of the water type, pharmaceutical water systems are routinely monitored to survey the physico-chemical, microbiological and endotoxin control. The purpose of water monitoring system is to ensure the followings:

1. The stability and process control of all the water generation equipment, storage, and distribution systems and quality control to consistently manufacture the intended quality of water that consistently meets the microbiological, bacterial endotoxin, and chemical purity of the water.
2. Ensure that the critical parameters of controls are adequate to handle minor process drifts.
3. Provide the assessment of the effectiveness of the procedures for maintenance, cleaning, and sanitization procedures.
4. The monitoring data is capable of identifying adverse trends and early warning on deviations from a state of control and contamination build-up.

Sampling and frequency of monitoring

The sampling and frequency of monitoring of the water systems should be sufficient to ensure that the water quality meets its specifications for its intended use.

For water systems, monitoring for microbial and endotoxin is critical because the potential for microbial build-up in the system is high, especially in ambient water, since some microorganisms can survive and proliferate. In contrast to ambient water systems, the hot circulating loops are less prone to microbial build-up unless the system is poorly designed.

The frequency of monitoring and sampling sites selection depends on the type of water and manufacturing process, system configuration and facility design. No single sampling scheme is appropriate for all types of water. In addition, changes in sampling frequency, whether temporary or permanent, may be required based on changes in system configuration, compendial requirements, or development of significant trends. Also the sampling frequency plan should be designed in such a way that allows detecting changes in chemical purity, bacterial endotoxins test (BET) and microbial counts due to seasonal variations as appropriate depending on the type of water.

The followings points should be regarded for reducing frequency of testing. Sampling location and type of testing must be determined as per the following criteria:

1. Regulatory agencies require that WFI system should be tested daily.
2. To identify the overall system control requirements pre-assessment of the criticality of the ports based on the risk assessment of the system design prior to
characterization
3. For routine monitoring of the adequately characterized and validated water systems, the site and frequency selection can be reduced based on risk analysis that includes the assessment of the initial validation data and considerable historic data to justify the frequency of sampling/testing based on the criticality of the ports. The critical ports must be tested daily. In addition, the ports used to obtain water for manufacturing should be tested on the day of use.
4. Criticality of water system performance
5. The ports can be divided into two broad categories; critical and non-critical ports. Critical ports allow the verification of the overall system performance, some examples of critical ports are return to tank, first point and the last point in the loop.
6. Nature of the manufacturing process
7. The intended use of the water in the manufacturing process
8. The water type

These points are described in further details below.

A key goal of proper selection of sampling location and monitoring frequencies is to identify potential system deficiencies. There are many considerations in establishing appropriate sites for sampling (e.g., facilities design, system configurations, validation data, process, historical data, test methodology, etc.). To demonstrate overall system control, one approach that may be considered is to divide the sampling ports into two broad categories critical and non-critical based on the system design, pre-characterization risk assessment and intended use of the water system. During system characterization and validation phase the entire system is validated and characterized. Post-validation for routine monitoring the critical point of use (POU) sampling ports should be tested at more rigorous frequencies than the other non-critical POU ports in the loop which may be tested relatively less frequently. Regardless of the validation, critical system ports should be tested daily. This approach allows verifying the systemic issues related to microbial contamination. Some examples of the critical ports are POU, sampling ports, such as supply and return to tank, after pump, feed to clean steam generator, and hot WFI stills. The water ports used for manufacturing should be tested on the day of use. The testing of critical POU assures that all critical equipment operate within design specification to produce desired quality water. Such testing also helps in resolving the local port problems when the other non-critical POU show excursions. The failures or an adverse trend of recovery of organisms at multiple critical ports indicates systemic issues. The frequency of the non-critical ports can be adjusted according to validation.

Once a water system is validated to be in a state of control, appropriate samples should be taken from the holding and distribution system to assess the microbiological quality for its intended use. In general, the site selection and frequency adjustments should be justified using risk-based approaches, historic and validation data.

The site selection and frequency adjustments should be justified using risk based approaches depending on the type product and manufacturing process; for example non-sterile solid dosage forms can be manufactured using purified water whereas the sterile parenteral manufacturing process require WFI manufacturing. The sampling frequency should be rigorous for parenteral manufacturing than that required for non-sterile products. For biotechnology manufacturing process for the manufacture drug substance requires stringent assurance for WFI quality which is similar to parenteral drugs.

The sample collection and testing requirements as representative product lot manufactured are described below.

Sample Collection and Testing

Water samples should be collected in a manner that is consistent with manufacturing practices. For example, if manufacturing flushes the POU prior to use, it is appropriate for samples to be collected with the same flush cycle. On the other hand, if manufacturing does not flush POU, there should be no flush prior to sample collection. It is recommended to sample through hoses and not directly from the tap if manufacturing practices require the use of hoses. The sampling from leaking taps should be avoided. Leaking taps should be repaired prior to use for processing and testing. Carefully choose distribution system sample locations to demonstrate microbiological quality throughout the distribution system.

Personnel responsible for sample collection and testing the water systems must be trained on the overall good aseptic techniques for sample collection and test methods/procedures. The Standard Operating Procedures (SOP) must describe the validated sampling and testing procedures. In addition they should include the following:
1. Sampling Schedule
2. Map of Sampling Sites
3. Required Alert and Action Levels

Table 1 shows that the type of media used incubation conditions used for sample testing and limits for various types of water. For purified water the standards do allow to test 1 mL sample volume because the limit is 100 CFU/ml. However, differences in the recommended sample volume for WFI testing exist in various compendial documents; for example USP requires that 100 mL of the sample be tested while EP and JP requires 200
Critical parameters that directly affect product quality must be defined and routinely monitored. For water systems producing compendial bulk waters, the chemical and bacterial endotoxin specifications in the compendial monographs constitute the critical parameters. Although a bioburden specification is not listed in the water monographs, the microbial quality of water systems is viewed as a critical parameter that must be monitored, even though it is difficult to react to the results as microbial quality cannot be monitored in real-time. To address this issue, companies establish alert and action levels as system control parameters and evaluate routine water monitoring data against these levels.

**Sampling Program**

The frequency of testing must be sufficient, and samples should be taken from representative locations in the distribution system in order to demonstrate that the water system is in a state of chemical and microbial control. Typically, the frequency of testing and sampling sites are established on the basis of data generated during validation studies. In the PDA Technical Report No.13 Appendix C (PDA, 1990), the following testing frequencies for clean utilities are recommended:

1. Potable water: Weekly/microbial count and coliform testing
2. Purified water: Daily (when in production)/chemistry and microbial testing
3. Water for injection: Daily for feed-water to still/mol

**TABLE 1.** Guidelines for microbiological testing of various types of water by traditional procedures

| Method conditions               | Source (Potable) Water | Purified Water | Water for Injection |
|---------------------------------|------------------------|----------------|---------------------|
| Methods and Test Volumes        | Pour plate method-     | Pour plate method- | Membrane filtration 100 |
|                                 | minimum sample 1 ml    | minimum sample 1 ml or | ml (USP) or 200 ml   |
|                                 |                        | membrane filter 100 ml | (EP, JP)             |
| Tests and Incubation conditions | Plate Count agar:      | Plate count agar:30-35°C for 2-3 day | R2A agar-30-35°C for |
|                                 | 30-35°C for 2-3 day    | for 2-3 day minimum | 5-7 days minimum     |
| Acceptance Levels:              | ≤ 500 cfu/ ml          | ≤ 100 cfu/ml     | ≤ 10 cfu/100 ml     |

**TABLE 2.** Differences in the growth promoting microorganisms recommended by EP and JP

| Medium                      | Microorganisms                  |
|-----------------------------|---------------------------------|
| R2A Agar                    | *Bacillus subtilis* ATCC 6633 | *Methylobacterium extorquens* NBRC 15911 |
|                             | *Pseudomonas aeruginosa* ATCC 9027 | *Pseudomonas fluorescens* ATCC 17356 |
| Standard Plate Count Agar   | NA                               | *Staphylococcus aureus* ATCC 6538 |
|                             | NA                               | *Escherichia coli* ATCC 8739 |

mL (Table 1). Due to these differences it is recommended to test the most stringent volume.

Microbiological examination of water should be initiated as soon as possible after collection of the sample; in accordance to validated procedures. If immediate processing is not possible, refrigerate samples at 2-8°C upon receipt in the laboratory. Time elapsing between collection and examination should not exceed 12 hours. Longer sample storage time should be validated.

Culture medium used for microbiological testing of purified water and WFI, should be capable of detecting and enumerating the aerobic, mesophilic microbial flora including those that require low nutrient media such as R2A regardless of the type of product manufactured. The R2A media should be released using the scheme described in compendial documents.

The difference in the compendial procedure for the growth promotion testing and organisms for the testing of R2A exists. JP requires that the organisms required for and organisms used of R2A agar should be conditioned in sterile purified water for at least 5 days at 20-25°C.

Differences in organisms required for growth promotion of the media by EP and JP are shown in Table 2.

**TESTING OF PHARMACEUTICAL WATERS**

Water systems are a significant part of regulatory quality inspections, and companies must routinely monitor the bulk water produced to ensure the chemical and microbial quality of this key pharmaceutical ingredient. Critical parameters that directly affect product quality must be defined and routinely monitored. For water systems producing compendial bulk waters, the chemical and bacterial endotoxin specifications in the compendial monographs constitute the critical parameters. Although a bioburden specification is not listed in the water monographs, the microbial quality of water systems is viewed as a critical parameter that must be monitored, even though it is difficult to react to the results as microbial quality cannot be monitored in real time. To address this issue, companies establish alert and action levels as system control parameters and evaluate routine water monitoring data against these levels.
microbial, chemistry and endotoxin; daily for return loop/chemistry and endotoxin; weekly rotation for all use points/microbial testing
4. Clean steam: Monthly/chemistry and endotoxin testing

**Sample Collection and Preservation**

Collection bottles for microbial testing must be sterile, and sampling must be performed using aseptic technique. When performing sampling for a type of water that contains residual chlorine or other halogens, a reducing agent must be added to the containers prior to water collection. Sodium thiosulfate at a concentration of 0.1 mL of a 10% solution per 120 mL of sample has been proven satisfactory for neutralizing chlorinated water.

For sample collection, sufficient headspace should be present in the bottle to facilitate mixing prior to testing. This is critical because microbial contamination is not uniformly distributed in a sample. When collecting bulk water at the points of use, the operator must flush the line, allowing a forceful flow of water for about 1-3 min prior to sampling. This will ensure that the sample collected reflects the quality of the water in the system. If a sampling hose is normally used to procure water for manufacturing purposes, the operator must not remove the hose when sampling the water for testing. This procedure will ensure that the sample collected is representative of the water used in production and that any biofilm formed in the hose is detected.

If samples cannot be processed within 1 h after collection, they must be stored under refrigerated conditions (2-8°C) until testing is performed. For most accurate data, samples should be processed within 1 h after collection (APHA, American Public Health Association, 2005). If this is not possible, they should be kept refrigerated, ideally for a maximum of about 12 h, and not exceeding 48 h (USP, 2008). The analysts must be aware that any delay in testing may impact the test results because microbial viability of potential contaminants may decrease, or in some cases increase, if samples are not stored properly.

**Bioburden Testing**

The bioburden of waters is evaluated on the basis of the number of CFUs in a fixed sample volume tested. Microbiological testing of drinking water (potable water), which is used as the main source water (feed water) in pharmaceutical manufacturing facilities, is subject to the National Primary Drinking Water Regulations (NPDWR) issued by the EPA. For pharmaceutical-grade waters, there is no official standard recovery method; however, the USP recommends, in the informational Chapter <1231>, the following recovery methods that are derived from the Standard Methods for the Examination of Water and Wastewater (APHA, 2005):

1. **Drinking water**
   - Use pour-plate method
   - Test a minimum of 1.0 mL of sample
   - Use plate count agar
   - Incubate at 30-35°C for a minimum of 48-72 h

2. **Purified water**
   - Use pour-plate or membrane filtration method
   - Test a minimum of 1.0 mL of sample
   - Use plate count agar
   - Incubate at 30-35°C for a minimum of 48-72 h

3. **Water for injection**
   - Use membrane filtration method
   - Test a minimum of 100 mL of sample
   - Use plate count agar
   - Incubate at 30-35°C for a minimum of 48-72 h

Although the bioburden methods recommended by the USP Chapter <1231> are not always ideal documents for the detection of stressed, injured, viable but non-cultural (VBNC) and starved organisms, they are still recognized as appropriate techniques for establishing trends in bioburden in water systems in a timely manner. The USP also states that other recovery methods, including media and incubation conditions, and larger sample volumes may be used for the optimal recovery of microorganisms found in various types of water systems. In fact, most highly purified water systems are extremely effective in the removal and prevention of biofilm formation; thus, a sample size of 1.0 mL is not appropriate for testing and trending the microbial quality of the water produced.

When using sample volumes larger than 1.0 mL, the membrane filtration method should be used; a membrane filter with a rating of 0.45 or 022 µm is generally the preferred method for testing liquid samples for bioburden. This is especially true for water samples because the filtration process allows retention and recovery of a high number of small cells (e.g., Gram negative and starved microorganisms) typically found systems. Based on the expected bioburden of the samples collected, most pharmaceutical companies have chosen the membrane filtration method for testing purified waters and the pour-plate method, using a 1.0 mL sample volume, for the testing of feed water. Water samples are processed using sterile filter units that combine a funnel and a grid membrane filter in one device and plated with agar-based media contained in ready-to-use cassettes.

**Recovery Media**

The plate count agar medium recommended by the USP for bioburden testing is also known as a standard
method agar, or tryptone glucose yeast agar (TGYA, or soybean casein digest agar, SCDA). This is a high-nutrient medium and may not be suitable for the recovery of many waterborne organisms that are considered starved, injured, VBNC and stressed. For the recovery of this type of bacteria in water, it is recommended that a low-nutrient medium such as R2A medium should be recommended. The validation of the selection of medium requires scientific rationale; in general, R2A agar seems to be popular and yields good results for isolating and detecting waterborne bacteria compared with SCD medium. R2A is a low-nutrient medium used for pour-plate, membrane filtration, or spread-plate methods. Some studies performed to compare microbial recovery from water samples using different types of media have indicated that R2A agar often yields higher counts as compared to high-nutrient media such as TGYA, TSA, and m-HPC agar (formerly called m-SPC agar) that are more appropriate for the general isolation and enumeration of heterotrophic and mesophilic bacteria (Robert, A.G. et al, 1991). The recommended bioburden methods that use high-nutrient media such as SCD medium specify incubation conditions at 30-35°C for 48-72 h, but this procedure is not generally well validated. Therefore, when a low-nutrient medium like R2A medium is used, the test plates incubate at 20-25°C for 5-7 d. The longer the incubation and the lower the temperature, the higher the counts obtained because these incubation conditions improve the recovery of waterborne and slow-growing organisms of stressed, injured, viable but non-culturable (VBNC) and starved organisms in water.

The best recovery medium and incubation conditions for testing of water samples has been a hot topic for many years. Although it is widely known that most methods used cannot recover the invisible microbial flora found in water systems and that bioburden counts recovered are nothing but a rough estimate of the microbial quality of the water produced, scientists often debate the value of having a higher baseline count at the expense of longer test turnaround times. The reality is that detecting higher microbial counts may not add value to early detection of an excursion or an adverse trend. Many of the so-called starved organisms and slow growers become nonviable upon subculturing and therefore cannot be characterized or require resuscitation prior to further characterization. Reviving microbial cells can be accomplished by inoculating the isolated organism in a liquid culture medium and incubating at a moderate temperature. However, this approach adds to the overall testing turnaround time, and may not be practical or considered a value-added activity.

The consensus is that each company should generate data to support the best methodology for testing the microbial quality of its water systems. The decision to use one methodology over another must be based on the company’s needs and knowledge of the water system. The waterborne sample to be tested must be the same for the both companies, otherwise it is simply the inter-laboratory tests. A company may prefer to use the method for attaining a better result of the microbial quantity test of the water system. Most companies seem to opt for methods that yield the highest microbial recovery in the shortest amount of time, which is however hard to attain. Verification of the best methodology for a given water system can be performed following experimentation with alternate recovery approaches during or prior to validation of a new water system. In addition to this initial method suitability study, periodic reassessments may be needed for new water systems as the microbial flora gradually stabilizes relative to the original flora detected during system validation.

Identification of Waterborne Microorganisms

Information on the types of microbes found in water systems is helpful in identifying the source of contamination. Knowing the typical microbial flora in a water system aids in the evaluation of the effectiveness of system sanitization and in personnel training; it can also serve in early detection of system deterioration.

During system validation, it is a good practice to identify representative isolates in order to establish a baseline microbial flora in the water system. It is also recommended that some of the frequently isolated microorganisms be maintained in the quality control (QC) laboratory culture collection to be used in studies such as sanitizer efficacy and product bioburden suitability testing.

During routine monitoring of water systems, every isolated colony as far as possible needs to be identified. Microbial identification should be performed to provide information for trending purposes and also to assist in manufacturing investigations in case of product contamination.

As a general guideline, when the bioburden detected exceeds the alert level, representative colonies are gram-stained to evaluate the possible source of contamination. This simple technique provides for microscopic observation of cell morphology (i.e., coccus, rod, single cells, chains, clusters, etc.) as well as for a gram reaction so that the isolate can be classified as either gram-positive or gram negative.

There are other simple techniques such as the oxidase test and the catalase test as well as checking for sporulation in gram-positive rods that can be useful for preliminary microbial identification. For most investigations into microbial excursions, these techniques provide sufficient information for data trending purposes. For example, most water isolates are gram negative.
rods, and many are oxidase-positive (e.g., Pseudomonads). However, if gram-positive cocci, which are typically human-borne are found in a water sample, it may be an indication of poor aseptic technique applied during sample collection or sample testing. In many cases, Bacillus organisms are isolated from water samples. Such events, although common occurrences in some companies, should be rare, and when they occur, it could signal poor sampling technique, insufficient flushing of sample ports, or laboratory contamination because Bacillus sp is not waterborne microorganisms. In addition, any bacterial contamination found in a hot-water system should be suspected and investigated as a potential sample contamination or poor system maintenance.

When the number of recovered organisms exceeds the action level, it is expected that identification to the genus and species level was done and an investigation performed. There is a chance that the action level excursion could be due to sampling error or inadequate port flushing. However, if there is an indication of true sample-point contamination, the identity of the microbial isolate will be critical for an evaluation of potential product impact.

**ESTABLISHING ALERT AND ACTION LEVELS**

While establishing alert and action levels for bioburden in pharmaceutical-grade waters, factors such as the intended use, the nature of the product being manufactured, and the effect of the manufacturing process on the fate of viable organisms should be taken into account. For purified water and WFI, chemical and endotoxin (WFI only) specifications are clearly defined in the pharmacopeia. However, there are no specifications for microbial quality. In lieu of limits/specifications, alert and action levels are established based on system capability and as process-control indicators.

When establishing alert and action levels, one must recognize the difference between design range and operating range. For example, a purified water system may be designed to deliver water that meets the compendial bioburden guideline of not more than 100 CFU/mL. However, based on company needs and products manufactured, action levels may be set at much lower microbial levels to reflect the allowable operating range that will assure the quality of the final product manufactured. In order to further ensure proper system maintenance and control, a company may yet choose to set alert levels to reflect the normal operating range of the system. Exceeding an alert level should be interpreted as a warning that the system may be drifting away from a state of microbial control. These events do not necessarily require corrective actions. Exceeding an action level should require an immediate investigation into the event so that appropriate corrective actions can be taken to bring the system back into a state of microbial control.

A pattern of multiple and frequent alert level excursions should be treated as an action level excursion and appropriate corrective measures must be taken. Types of immediate actions to take when results exceed action levels often include system sterilization, identification of organisms isolated, evaluation of the possible adverse product impact, and further sampling and monitoring of the water supply as well as other sampling points in the distribution loop. One point to remember is that, although an action level may be exceeded and corrective measures taken, it does not necessarily mean that this raw material is unsuitable for use. This decision will be based on the outcome of the investigation performed to assess the quality of the water produced.

Process controls for water systems may also involve qualitative/quantitative limits, such as the absence of a particular microbial species. Some companies may choose to monitor specified organisms that are known to cause problems to production equipment through formation of biofilms or compromise the manufactured product. In most cases, companies establish acceptable levels on the basis of the type of microorganisms and the number of colonies detected as the negative impact of a particular microorganism is often greater if present in high numbers.

Besides taking action when alert or action levels are exceeded, a company should establish a system to trend the water-monitoring data for detection of adverse trends. Data that show a deterioration of the microbial quality of the water system over time require attention in determining the cause and in the implementation of corrective measures. In addition, because alert and action levels should be based on historical data, it is common practice to reevaluate/recalculate these values on an annual basis. In fact, diligent evaluation/interpretation of, and prompt reaction to, data collected are key aspects of an effective management program for water systems.

There is no true consensus on the best approach of setting up alert and action levels based on historical data. The PDA Technical Report No. 13, Fundamentals of an Environmental Monitoring Program (PDA, 1990), describes the following approaches:

1. Cut-off approach: This method uses the last 100 data monitoring points and uses the 95th and 99th percentile values as the alert and action levels.
2. Normal distribution approach: This method calculates the alert level as the mean plus two times the standard deviation (2SD, 2 standard deviations),
and the action level as the mean plus three times the standard deviation (3SD) of a population of data points. This method suits a population with high microbial counts best. For low counts, a Poisson distribution should be used.

3. Nonparametric tolerance limits approach: Given the fact that clean utilities data (and environmental monitoring data) are not normally distributed and in most cases skewed towards zero counts, non-parametric (distribution-free) statistical methods seem more appropriate for data trend analysis. Nonparametric statistical methods are also simpler and often involve less computational work. Examples of such methods include the Kruskal-Wallis Analysis of Ranks and the Median Test. To set up alert and action levels, the PDA document recommends using the tolerance limits (TL) approach. TL differs from confidence intervals in that they provide an interval within at least a proportion “X”. A proportion “X” of a population lies within a certain probability that the stated interval does indeed contain that proportion “X”. For the alert level, TL can be set at a probability value (P) equal to 0.95 and a Gamma (γ) coefficient (also a probability value) of 0.95. For the action level, TL can be set using a γ value equal to 0.95 and a P value equal to 0.99.

When choosing the best statistical method for data trending, it is important that the user understands the differences among the tools available and how they apply to the set of data being evaluated.

One important point to remember is that the values obtained and the historical data collected are directly related to the type of bioburden methodology chosen for monitoring the water systems. If a company chooses to change the recovery media or even the technology used for detection of microbial contamination, it will have an impact on the test results obtained and how they relate to the established alert and action levels.

VALIDATION OF WATER SYSTEMS

The suitability and performance of water systems to produce water of acceptable chemical and microbiological quality must be validated prior to its use in the production of pharmaceutical products. Validation comprises commissioning and qualification activities that should ideally start with system design qualification (DQ). A well-designed water system has a great impact on its longevity, ensuring optimum operation and minimum routine maintenance costs. System design should address the pretreatment and final treatment of the water, the storage and distribution loops, as well as operation, maintenance, and sterilization procedures. Poorly designed systems having areas of stagnant conditions (dead legs), areas of low flow rate, poor-quality feed water, inadequate sterilization programs, and less-than-adequate material for construction will have an impact on validation efforts and incur long-term maintenance costs to the company. In addition to DQ, a water system validation program qualifies and documents system installation (as IQ), system operation (as OQ), and system performance (as PQ).

The performance qualification (PQ) activities for water systems are unique as they require monitoring of the system over a long period, typically lasting 12 months. Although few problems with the chemical quality of the system are observed over time, variations in the microbial flora as a result of seasonal changes in the feed water are often noted, thus adding to the challenges of validating a water system. The sampling program for a water system PQ consists of three successive phases:

1. Phase one begins after the water system is deemed fully operational following operational qualification (OQ) activities. During phase one, intensive daily sampling of major process points as well as the supply and return points take place for at least one month. This initial collection of data is valuable and usually sufficient to establish the acceptability of the water system. Data generated can be used to establish system-operating ranges and to create standard operating procedures, to include preventative maintenance and sterilization procedures. At the completion of phase one, the water may be released for use at risk or with limited applications, or both.

2. Phase two of the validation starts at the completion of phase one, and it may last for another month or two. During this phase of the study, the same testing frequency as well as the number and location of points sampled during phase one are maintained. Phase two is designed to demonstrate consistency in system performance and production of water of a specified quality. At the conclusion of phase two, if all test results are acceptable, the water system is considered validated and is released for use in production without restrictions. However, extended performance evaluation, especially to account for seasonal variations in microbial population of the feed water, is needed. This is accomplished during phase three of the validation activities.

3. Phase three ensures that additional and frequent monitoring is performed during the first year of the water system’s operation. This additional qualification activity is performed to gather sufficient data for trending purposes and for setting meaningful alert and action levels. Typically, the number and frequency of sampling is reduced to reflect use and critical sampling sites. Once the data from the first year of operation are obtained, alert and action levels,
previously established based on limited historical/baseline data or perhaps based on a combination of equipment design capabilities and compendial guidelines can be reevaluated.

Upon completion of a water system validation, routine monitoring, preventive maintenance, and sterilization must continue in order to control the microbial quality of the water produced.

From a microbiological perspective, the validation of water systems includes testing for bioburden (heterotrophic counts of mesophilic microorganisms), bacterial endotoxins (WFI and pure steam) and, if applicable, screening for organisms of concern. In the past, there was an expectation that hot-water systems should be monitored for thermophiles. However, over the past few years, there has been a shift in paradigm and a realization that the idea that thermophilic bacteria thriving in hot purified water systems is a misconception. The reality is that a hot purified-water system is an extremely hostile environment for these types of microorganisms. Thermophilic and hyperthermophilic microbes require unique environments for their survival and proliferation that include (in most cases) a specific redox potential, extreme pH conditions, temperatures above 70°C, and concentrations of carbon and minerals not found in pharmaceutical waters. For example, most Archaeans are chemolithoautotrophes, and the Bacillus spp. (Geobacillus stearothermophilus, Bacillus brevis, and B. acidocaldarius), Clostridium spp., and Thermus spp. that can grow at higher temperatures have special nutritional requirements not found in high-purified water systems. Even if only the spores of Bacillus or Clostridium organisms were present in the water system, they would not be able to germinate, and would eventually be removed or die off (Martinez, 2004). Therefore, the consensus nowadays among industry experts is that testing for thermophiles in hot pharmaceutical water systems is a non-value-added and costly activity.

The qualification of the bioburden methodologies used for routine water testing (choice of best recovery method) can be incorporated into the water system validation protocol. Typically, at least two criteria (different media and incubation conditions) are evaluated through concurrent testing. Sometimes, the water system is too clean to produce data of statistical significance. In such cases, spike studies using representative waterborne isolates can be performed in the laboratory. Upon data evaluation, if there is a difference of more than 0.3 log (internationally harmonized) recovery between the methods evaluated, the medium/incubation conditions chosen for routine testing should be the one that yielded the highest recovery of microorganisms in the shortest amount of time. If the difference is less than 0.3 log (harmonized), any of the evaluated methods should be considered suitable for the application.

Remember that the most important aspect of a water-monitoring system is to generate data for early detection of adverse trends. The expectation that a method will be able to detect every type of microorganism present in the water system is unrealistic. A company should strive to develop a bioburden testing program that produces timely results so that management can quickly react to adverse conditions.

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