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Review

Methods, Protocols, Guidance and Standards for Performance Evaluation for Point-of-Use Water Treatment Technologies: History, Current Status, Future Needs and Directions

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Abstract: It is estimated that 780 million people do not have access to improved drinking water sources and approximately 2 billion people use fecally contaminated drinking water. Effective point-of-use water treatment systems (POU) can provide water with sufficiently reduced concentrations of pathogenic enteric microorganisms to not pose significant health risks to consumers. Household water treatment (HWT) systems utilize various technologies that physically remove and/or inactivate pathogens. A limited number of governmental and other institutional entities have developed testing protocols to evaluate the performance of POU water treatment systems. Such testing protocols are essential to documenting effective performance because inferior and ineffective POU treatment technologies are thought to be in widespread use. This critical review examines specific practices, procedures and specification of widely available POU system evaluation protocols. Testing protocols should provide standardized and detailed instructions yet be sufficiently flexible to deal with different treatment technologies, test microbe priorities and choices, testing facility capabilities and public health needs. Appropriate infectivity or culture assays should be used to quantify test enteric bacteria, viruses and protozoan parasites, or other appropriate surrogates or substitutes for them, although processes based on physical removal can be tested by methods that detect microbes as particles. Recommendations include further research of stock microbe production and handling methods to consistently yield test microbes in a realistic state of aggregation and, in the case of bacteria, appropriately physiologically stressed. Bacterial quantification methods should address the phenomenon of bacterial injury and repair in order to maximally recover those that are culturable and potentially infectious. It is only with harmonized national and international testing protocols and performance targets that independent and unbiased testing can be done to assure consumers that POU treatment technologies are able to produce water of high microbial quality and low health risk.

Keywords: point-of-use treatment (POU); household water treatment (HWT); drinking water; water disinfection; bacterial injury and repair; enteric bacteria; enteric viruses; protozoan parasites; microbial indicators; performance evaluation; testing protocols; removal; inactivation

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1. Introduction and Background

It is estimated by the World Health Organization (WHO) that about 780 million people acquire their drinking water from unimproved surface, spring and ground water sources, and many more drink water from improved sources that are still contaminated

with pathogenic microorganisms [1]. The grim result of this situation is that diarrhea is the second leading cause of death of children younger than five, with an estimated 526,000 children dying each year [2].

While governments and nongovernmental entities attempt to provide community and household access to centralized and managed systems for continuous, sustainable and safe drinking water, an alternative temporary but immediate solution for safe drinking water access is the use of point-of-use (POU) water treatment. POU/household water treatment (HWT) and safe storage systems are devices or treatment processes typically designed to provide an adequate daily volume of treated drinking water for each household.

Treatment of drinking water at POU, typically in the household, is an ancient and established practice that continues today and is increasingly encouraged by international organizations, national governments and the private sector as a way to improve microbial quality of water that may be used for drinking, cooking, or personal hygiene in homes, schools, healthcare facilities, or other establishments. POU water treatment is also widely employed by travelers, field military operations, for emergency and disaster response and where and when other options to produce and deliver safe water are unavailable or compromised. Meta-analyses of the numerous studies examining the effectiveness of various intervention strategies to reduce diarrhea have found that at least some household POU treatment devices do improve drinking water quality and reduce the risk of diarrhea by an estimated 30% to 40% [3,4].

There are a number of specific POU/HWT technologies and various designs of do-it-yourself or commercial devices to reduce concentrations of microbes in drinking water [5–8]. The key technologies recommended and used for POU water treatment include chemical disinfection, SODIS (solar disinfection, heat or heat plus ultraviolet radiation), ultraviolet (UV) irradiation, chemical coagulation, filtration and combined or multi-barrier processes, such as coagulation-flocculation and disinfection. Descriptions of these technologies are provided in Table 1.

The absolute and relative microbial reduction performance and sustainability of these various POU water treatment technologies in households are compared in References [5,8]. The WHO includes POU/HWT systems in its Guidelines for Drinking-Water Quality and has created an International Network to Promote Household Water Treatment and Safe Storage of drinking water, along with many other participating organizations (see: https://www.who.int/water_sanitation_health/water-quality/household/household-water-network/en/, accessed on 15 January 2021). WHO has specified HWT performance evaluation targets based on health risk levels and corresponding $\log_{10}$ microbial reductions and developed and implemented a formal scheme to evaluate HWT technologies based on independent performance assessments.

However, a variety of specific factors and conditions can influence the extent to which a POU/HWT technology reduces the concentrations of various microbes in water during performance evaluations. Such factors and conditions include the choice of test microbes, the specific sources and physical and physiological conditions of these microbes and their conditions or states in the waters being tested, the choice, characteristics of the test waters, the specific conditions and frequencies of challenging the treatment technology with microbe-laden water in a test protocol and procedure, the procedures used to detect and quantify the test microbes in the challenge water and the treated water and the representativeness of the testing conditions and procedures relative to those likely to prevail in actual practice when used by people in their households or other POU locations.

Table 1. Key technologies used for point-of-use (POU)/household water treatment (HWT).

Technology Type	Description and Related Features
Chemical Disinfectants	
Halogens and Other Oxidants	Free chlorine is most widely used to kill or inactivate microorganisms in water. It is used at POU as hypochlorous acid or sodium dichloroisocyanurate. Other POU halogens are chlorine dioxide, iodine and bromine. Peroxides and ozone disinfectants of water are available but are not recommended for POU/HWT treatment for pathogens.
Antimicrobial Metals	Silver or copper are available for POU disinfection but are weaker and slower acting than oxidants and require longer contact times, such as water storage.
Radiation	
Solar Radiation	Inactivates or kills microbes by a combination of germicidal ultraviolet (UV) radiation and heat. It is promoted as a do-it-yourself (DIY) technology in which people and communities put water in clear plastic bottles and expose them to direct sunlight for a day or more to achieve microbial reductions by the actions of UV radiation and heat; alternatively, water put in cook pots and exposed to solar radiation collected by solar reflectors can heat sufficiently to pasteurize. Commercial devices are also available.
UV Irradiation from lamps	UV lamps that emit radiation in the germicidal range as either monochromatic or polychromatic radiation inactivate or kill microbes in water.
Coagulation-Flocculation for Microbial Removal from Water	Addition of inorganic or organic chemical coagulants to water results in the formation of chemical precipitates that attract and capture microbes and other colloidal particles for subsequent removal by sedimentation or filtration. Slow mixing of coagulated water encourages the formation of larger coagulated floc particles (flocculation) that enhance particle removal from water by gravity settling or filtration processes.
Filtration	Porous filters in the form of fired ceramic clay candles or pots, granular media filters, such as those containing sand, other mineral particles or granular carbon, membrane filters of various pore sizes ranging from microfilters to ultrafilters and cloth/fabric/fiber/paper media filters will remove microbes from water if pore sizes are small enough to retain them and thereby strain them out. Microbial removal is primarily a physical-chemical process based on size exclusion and related physical-chemical retention phenomena (interception, sedimentation, adsorption, ion exchange, etc.)
Granular Media and Other Biologically Active Porous Media Filters	Slow sand filters and biosand filters operate under conditions that promote the development of biological activity on the surfaces of the filter media to create a biologically active filter matrix in which microbial contaminants are physically removed and degraded by the biological activities present (adsorption/absorption, predation, enzymatic degradation and biological digestion).
Dual or Multi-Barrier Technologies	By combining two or more of the aforementioned treatment technology processes or sometimes others, devices or systems are created that provide more treatment processes for removal or inactivation of microbes in water. Examples are combined coagulant-flocculant-chemical disinfection technologies, filtration-chemical disinfection systems and filtration-UV irradiation systems.

Given the worldwide need for effective HWT devices and the wide variety of POU treatment options and designs, there is also a need for POU testing protocols and methods which provide objective and accurate assessments of the microbe removal and inactivation efficiency of these devices under testing conditions that are representative of real life, field situations and as far as practically feasible, simulate environmental water use conditions, water qualities and the types, conditions and physiologies of the microorganisms of concern and interest. The testing protocols need to provide data that can support established standards deemed acceptable to public health regulatory officials, POU device manufacturers and users worldwide, yet be flexible enough to be adaptable to local conditions and testing resources. A survey by the WHO of mostly developing countries found that less than half of the responding countries regulate HWT devices used or sold in their country [9].

The focus of this review is on the reduction of microbial contaminants in water intended for drinking and other potable purposes because waterborne infectious diseases continue to be of the greatest global health concern in drinking water due to their immediate risks of morbidity and mortality. While chemical contaminants, such as arsenic and fluoride, are also of health concern, the health effects from drinking water exposures to these chemicals are typically the result of daily waterborne exposures over long periods of time. Furthermore, most policies, guidance, regulations, protocols and procedures have focused exclusively or primarily on evaluating performance in reducing concentrations of various microbial contaminants in drinking water.

The specific purposes of this critical review are to: (1) assess the current status and landscape of POU technology performance evaluation and verification procedures available internationally, nationally and perhaps locally, (2) consider the specific requirements, recommendations, protocols, conditions and procedures employed in the available standards, guidelines, regulations, recommendations and protocols for POU technology performance evaluation and verification, (3) identify limitations, gaps and other deficiencies in these available resources and (4) propose ways to improve and harmonize POU household technology performance evaluation and verification.

Specific POU/HWT test protocol-related issues to be addressed in this document are: (1) adaption of test protocols to different microbial reduction treatment technologies, (2) basis for microbe reduction performance standards, recommendations and guidelines, (3) consideration of the options for physical removal of microbes or loss of their infectivity by the technology, (4) choices of and specifications for test microbes and their sources, (5) species and strain variation among test microbes, (6) conditions or states of test microbes (such as, degree of aggregation, adsorption to or encapsulation in particulate matter, physiological condition or state, stress or injury), (7) microbial assay methods, and in particular the use of injury repair and other resuscitation culture methods and materials for enumeration, (8) test water characteristics and the materials used for adjustment of quality, (9) testing schedules, (10) safe storage and handling of product water and (11) measurement of other product water parameters indicating performance of a POU system.

The existing POU testing protocols to be reviewed in this document are: (1) The 1987 US Environmental Protection Agency (EPA) Guide Standard Protocol and its descendants or derivatives, (2) 2014 National Sanitation Foundation (NSF) Protocol P231 Microbiological Water Purifiers, (3) 2012 NSF Protocol P248 Military Operations Microbiological Water Purifiers, (4) 2020 NSF/ANSI Standard 55 Ultraviolet Microbiological Water Treatment Systems, (5) 2020 NSF/ANSI (American National Standards Institute) Standard 62 Drinking Water Distillation Systems, (6) 2020 NSF/ANSI Standard 244 Drinking Water Treatment Units—Supplemental Microbiological Water Treatments, (7) the China Ministry of Health GB/T 5750-2006 Standard [10], (8) the Chinese protocol Guideline for Declaration of Health Administration Permission for Products Relating to Health and Safety of Drinking Water [11], (9) the Brazilian NORM ABNT NBR (Associação Brasileira de Normas Técnicas) 15176:2004, and (10) the Mexico Standard NOM (Normas Oficiales Mexicanas)-244-SSA1-2008 and (11) Water Quality India Association (WQIA) Protocol IP 100: Guide Standard and Protocol for Microbiological Evaluation of Drinking Water Treatment Devices In addition,

we review the 2012 implemented International Scheme to Evaluate Household Water Treatment Technologies of the World Health Organization [12].

Methods

For this review we have repeatedly examined the available scientific and technical literature on POU/HWTs and its performance evaluation since the 1970s, at least annually. Our most recent review of the literature was through 2020 and included the literature in PubMed, BioMed Central, Google Scholar, Science Direct and Web of Science.

We have acquired and reviewed articles and other communications on advances in science, technology, engineering, policy, practice and knowledge in HWTS since the 1970s. We have also consulted and communicated regularly with HWTS experts, including HWTS performance evaluation experts at WHO, US Centers for Disease Control (CDC), US EPA, other national government agencies, dozens of Non-governmental oganizations NGOs and other relevant organizations such as NSF-International and the American Society for Testing and Materials (ASTM), as well as many private sector companies who make and market HWTS products.

We have also organized, co-organized, facilitated and participated in many Household Water Treatment and Safe Stoage (HWTS) meetings, conferences and workshops in the last 20 years that address HWTS performance evaluation, programs, schemes, guidance, recommendations and standards. Specifically, we have helped organize and participated in annual meetings on HWTS and its performance evaluation as sponsored by the World Health Organization, going back to the year 2000 when the WHO initiated its global efforts on HWTS. More recently, we have been involved with HWTS and its performance evaluation through the University of North Carolina Water Institute and its annual "Water and Health Conference: Where Science Meets Policy", in partnership with the World Health Organization and other stakeholders, beginning in 2010. In addition, we have helped organize and participated in other periodic international meetings, such as the biennial Health-related Water Microbiology Symposium of the International Water Association, other periodic HWTS meetings sponsored by the World Health Organization, other International Water Association meetings, conferences and workshops and the Water Environment Federation, as examples.

Our most recent review of the literature was in 2020 and included the relevant literature in PubMed, BioMed Central, Google Scholar, Science Direct and Web of Science, which were searched systematically using key search words and search strings.

Contributors to this review include scientists who research and otherwise study and practice HWTS, its performance evaluation, policies and procedures since the 1970s. These efforts include collaborations with authoritative subject matter experts at entities such as WHO Pan American Health Organization (PAHO) region, US EPA, US CDC, NSF-International, ASTM, other international agencies, numerous NGOs, academic institutions and the private sector. We have authored or co-authored previous reviews on HWTS and its performance evaluation, including seminal WHO documents such as: "Managing Water in the Home: Accelerated Health Gains from Improved Water Supply", WHO/SDE/WSH/02.07 (World Health Organizatin/Department of Sustainable Development and Healthy Environments/Water, Sanitation, Hygeine and Health) and "Evaluating Household Water Treatment Options: Health-based Targets and Microbiological Performance", ISBN 978 92 4 154822 9.

Another author of this document is a leading representative of NSF-International and its program on POU technologies and their performance evaluation and is also a key member of the WHO HWTS Performance Evaluation Scheme leadership and its management program. In addition, this co-author has a major role in leading NSF-International's POU/HWTS program for technology specifications and performance evaluation testing in partnership with the US military. The relationship between US EPA and NSF-International on guidelines and performance evaluation of POU water treatment technologies goes back to the time of the development of the 1987 EPA Guide Standard Protocol, when EPA en-

gaged with the organization to develop this guidance, beginning in 1984. NSF-International also established a partnership with the World Health Organization on POU/HWT performance evaluation protocol development and management for technology testing in 2014, called the WHO "International Scheme to Evaluate Household Water Treatment Technologies" and they have jointly managed this program ever since.

The University of North Carolina at Chapel Hill (UNC), the current or previous affiliation of many of the authors, is a founding member of the WHO international Network on Household Water Treatment and Safe Storage, which was created in 2003. Subsequently, the UNC Water Institute has co-sponsored annual meetings of this Network at the UNC Water and Health Conference. Since the 1970s, numerous students, post-docs and research scientific and technical staff members led by co-author Mark Sobsey at UNC have engaged in POU/HWT research and development and performance evaluations for nearly all of the known treatment technologies, including chemical disinfectants, UV radiation and sunlight, various filtration processes and devices, chemical coagulation and flocculation technologies and multi-barrier treatment systems. Hence, this report is based on a comprehensive consideration of all of these information sources as well as our own research that has spanned 6 decades.

This document is the product of an effort that began in 2011, shortly after the publication of the WHO document "Evaluating Household Water Treatment Options: Health-based Targets and Microbiological Performance" and builds on the scientific and technical knowledge base that has developed since that time.

2. Point-of-Use Technology Evaluation Schemes, Sponsoring Entities and Evaluation Options

Currently, a number of organizations and some national governments provide recommendations, guidance, specifications, protocols and procedures or descriptions of methods and materials for POU performance technology evaluation. Foremost among these are the WHO, NSF-International, and the US Environmental Protection Agency (EPA), all of which now provide certification or some other type of formal performance evaluation approval process. Recognizing that supporting countries in developing and implementing national HWT and safe storage policies and programs is critical to sensitizing government and spurring action, there is an ongoing need to encourage and support such efforts.

Since 2011, the WHO has hosted regional workshops for ministries of health and water officials, implementers, manufacturers and researchers on POU and HWT technologies in East, West and Southern Africa and South and South-East Asia. One outcome of these workshops has been the establishment of national policies on HWT and safe storage by various countries, including Ghana, Kenya and Tanzania [13]. Additional countries, such as Vietnam, Nepal and Ethiopia, are in the midst of further developing and harmonizing policies, procedures, testing protocols, regulations and certifications for POU/HWT technology performance evaluation and approval.

Furthermore, the US EPA and other agencies around the world either have or are developing systems that provide for country/state-specific registration and use. That is, POU technology certification or approval may be specific to only that one country or have regional or international recognition. WHO [7,12], NSF-International [14–20], Water Quality Association-India [21] and the US EPA [22] have existing POU/HWT technology performance evaluation and certification or approval programs, and there are additional organizations who offer certifications based on these publicly available documents, such as the Water Quality Association (WQA), International Association of Plumbing and Mechanical Officials (IAPMO) and Underwriters Laboratory (UL).

POU/HWT units considered to be microbiological purifiers or making microbiological performance claims, at the request of the manufacturer, are tested and certified for compliance with the standards or alternative requirements of these certifying or approving entities.

POU technologies evaluated by independent entities follow prescriptive protocols to meet specified levels of performance for removal/inactivation of bacteria, viruses and

protozoan parasites (or surrogates) specified by these organizations. NSF-International P231 protocols evaluate performance according to the US EPA Guide Standard (1987). However, NSF P231 also includes non-performance-based provisions for material safety, literature and labeling. These documents have very specific and detailed testing performance specifications and numerical microbial reduction performance requirements for bacteria (6 $\log_{10}$), viruses (4 $\log_{10}$) and protozoan parasites (3 $\log_{10}$). However, these agencies also recognize that the testing protocols require some adaptation to the properties and characteristics of the specific technology being tested.

The WHO document "Evaluating Household Water Treatment Options: Health-based Targets and Microbiological Performance Specifications" [7], and a "WHO Recommendations" document define three different and quantitatively specific levels of technology performance, based on $\log_{10}$ microbial reductions, as described in a consensus publication [23]. However, that document does not provide a specific testing protocol and only provides general recommendations, descriptions of candidate materials and methods and suggestions for conducting technology performance evaluations that can be adapted to the conditions and contexts of different countries or other prevailing authorities. The document also encourages the adaptation of existing testing protocols and methods, where possible.

WHO announced a self-managed Scheme for the evaluation of HWT technologies in December 2012. The objective of the Scheme is to promote and coordinate independent and consistent testing for POU/HWT technology evaluation based on WHO criteria. To this end, WHO recognized the need for both technical advice and a detailed HWT technology performance evaluation protocol to be used by designated laboratories. WHO sought experts in drinking-water quality, microbiology and water treatment technology and regulation who demonstrated commitment to advancing public health goals and established an Independent Advisory Committee (IAC) to provide recommendations on technical aspects of the evaluation Scheme. The IAC provides advice to WHO on the criteria for the selection of testing laboratories, the evaluation and testing procedure, the harmonized testing protocols, the reporting templates for testing results and the evaluation of testing results for products submitted to the Scheme for evaluation.

Representative POU technology evaluation schemes or protocols that are either standards, guidance or recommendations are listed in Supplementary Table S1. The different key variables or factors relevant to their performance, evaluation conditions and specific materials and methods are listed for each entry. Some of these POU technology performance evaluation schemes or protocols are further described below.

The US EPA first developed a POU technology performance evaluation system for microbial contaminants in 1987, the Guide Standard and Protocol for Testing Microbiological Water Purifiers [22]. This document focused on the performance evaluation of filters (specifically ceramic filters), chemical disinfectants (specifically halogenated resin treatment units) and UV radiation disinfection units, although applicability to other POU technologies was indicated. The document specified microbial reduction performance requirements, specific test microbes, sources and preparation methods for test microbes, test microbe assay methods, microbe concentrations in test waters, specific compositions of test waters, specifications for design and use conditions of experimental rigs or setups and other specific test conditions and procedures. Appendix A of the Guide Standard (GS) protocol provides a Summary for Basis of Standards and Test Water.

The US EPA created a process by which individual manufacturers could apply for authorization to proceed with independent testing according to an EPA-approved protocol and, once testing was completed, have the testing results reviewed and assessed by the EPA for product registration. This testing and certification process is still in place and is the responsibility of the US EPA Office of Pesticide Programs.

From 2000 to 2013, NSF-International operated a Drinking Water Systems (DWS) Center dedicated to technology verification and, with assistance from the US EPA, managed an Environmental Technology Verification (ETV) Center that provided independent performance evaluation of drinking water technologies that aimed for a scale of produc-

tion/service for small communities, as opposed to households or individuals. The ETV tests and reports helped accelerate a technology's entrance into the commercial marketplace by providing consumers with verified results of product evaluations. According to the Center, "technologies were evaluated by a third-party organization, following technically sound test procedures, appropriate quality assurance/quality control (QA/QC) and a managed process, to provide purchasers, specifiers and permitters with credible and relevant data. Verification protocols were developed for specific technology areas following an open process with broad-based stakeholder input. The protocols then served as templates for developing test plans for the evaluation of individual technologies at specific locations. Verification reports detailing the results of the technology evaluations were publicly available to assist in marketing, purchase and permitting of the technologies. Verification statements, executive summaries of each verification test, were also provided." Although no longer maintained, test plans, protocols, verification statements and reports can be viewed at: https://archive.epa.gov/nrmrl/archive-etv/web/html/center-dws.html (accessed on 15 April 2021). They remain a valuable resource on POU/HWT performance evaluation.

While the EPA-ETV program ended in 2013, NSF continues to offer, under the Public Drinking Water Equipment certification program, ANSI/NSF 419 Public Drinking Water Equipment Performance—Filtration (NSF 2018). The scope of draft NSF 419 is to describe the performance evaluation test procedure for the product-specific, challenge testing of full-scale public drinking water ultrafiltration (UF) and microfiltration (MF) membrane modules, bag filters and cartridge filters for the removal of microbial contaminants. It will provide procedures to develop challenge-testing Log Removal Values, as required in US EPA's Long-Term 2 Enhanced Surface Water Treatment Rule (LT2 Rule) published in 40 CFR 141-subpart W.

NSF-International, accredited by the American National Standards Institute (ANSI), also has a global testing and certification program for drinking water treatment units, under which POU technology products can seek certification for an existing NSF/ANSI standard or an NSF Protocol. The ANSI process ensures standards are developed through a public process that brings balanced input from industry representatives, public health/regulatory officials and users/consumer representatives. There are NSF/ANSI standards, NSF protocol certification procedures for different POU technologies, including NSF/ANSI Standard 53, Drinking Water Treatment Units—Health Effects [19], NSF/ANSI Standard 58, Reverse Osmosis Drinking Water Treatment Systems [20], NSF/ANSI Standard 55, Ultraviolet Microbiological Water Treatment Systems [17], NSF/ANSI Standard 62, Drinking Water Distillation Systems [16], NSF Standard 244 Supplemental Microbiological Water Treatment Systems—Filtration [18], NSF Protocol P231, Microbiological Water Purifiers [15] and NSF Protocol P248, Military Operations Microbiological Water Purifiers [14]. It is important to note that some of these programs are intended for the evaluation of technologies to provide supplemental treatment to drinking water supplies, public or private. NSF/ANSI Standard 55, Class A systems, NSF P231 and NSF P248 include an evaluation of the technologies as primary treatment, with some caveats outlined in the document. Also, while NSF Standards 55, 62, 244 and NSF Protocols P231 and P248 evaluate performance for all three microbial groups (bacteria, virus, protozoan cysts), Standards 53 and 58 are primarily chemical reduction performance standards with the only microbiological claim being that of protozoan cyst reduction.

Like NSF-International and US EPA documents, WHO developed a document to address performance evaluation needs for HWTS. The WHO is a recognized global authority on public health, ideally suited to support and coordinate HWT evaluation efforts, and there is precedence for such coordination with its pesticide evaluation scheme and evaluation system for rapid malaria diagnostic tests. "Evaluating Household Water Treatment Options: Health-based targets and microbiological performance specifications" [7] is the WHO document that initially addressed HWTS performance evaluation as "WHO Recommendations," by providing specific performance targets for microbial reductions. However, unlike the former, the WHO document identifies three different microbial reduction perfor-

mance categories or levels, originally specified as "highly protective", "protective" and "protection" (initially "interim"). It specified $\log_{10}$ microbial reductions values for bacteria, viruses and protozoan parasites for each level of protection that were based on Disability Adjusted Life Year (DALY) values, with highly protective corresponding to 10^{-6} DALYS per person per year, protective corresponding to 10^{-4} DALYS per person per year and "interim" for technologies that were protective for 2 of the 3 categories of test microbes. While the WHO document on performance evaluation does not provide specific testing protocols, it does provide recommendations and guidance on testing procedures, materials and test conditions. However, development of specific test protocols and defined test conditions are left up to the user with encouragement to use existing test protocols, where possible.

With the input of an Independent Advisory Committee (IAC), the WHO Scheme designated testing facilities, and developed a more structured and formal POU technology performance evaluation program called the Scheme to Evaluate Household Water Treatment (HWT) Technologies, or "The Scheme" [12]. The Scheme allows POU technology providers to have their technology independently evaluated for microbial reduction performance against the WHO health-based criteria. If performance is found to be acceptable, such technologies shall be included in the list of WHO evaluated products on the Scheme website (https://www.who.int/water_sanitation_health/water-quality/household/scheme-household-water-treatment/en/, accessed on 15 January 2021). In implementing the Scheme, the three different performance categories identified in the WHO Recommendations document (e.g., highly protective, protective and interim) were changed to the performance tiers of: 3-star (★★★), 2-star (★★) and 1-star (★), based on the magnitude of their ability to remove viruses, bacteria and protozoa as $\log_{10}$ reduction values.

Results of the Scheme evaluation are intended to guide HWT product selection by Member States and procuring United Nations agencies. In this regard, the Scheme fills an important global and national need for independent health-based evaluation of HWT, especially considering the large number of product manufacturers, product claims and the limited capacity of low-income countries to conduct testing to verify these claims. The Scheme consists of four steps: (1) submission and assessment of product documentation, (2) product laboratory evaluation by a WHO assigned designated testing facility in accordance with the agreed testing procedure using WHO harmonized protocols and reporting templates for testing results and submission to WHO, (3) review of submitted performance testing results by an IAC that submits its review and recommendation to WHO and (4) WHO final determination of HWT technology performance based on evaluation results and IAC recommendation, with WHO listing of performance results for all technologies evaluated.

Round I technology evaluations under the scheme were reported in 2016 and Round II in 2018. At the time of this manuscript, Round III had just closed, with reporting planned for mid-2021. The international evaluation scheme and its management system are intended to ensure that WHO criteria are appropriately adopted, using a streamlined and harmonized process for evaluation of technologies used in many countries to provide an effective mechanism to build national capacity and provide global guidance.

WHO seeks to designate testing laboratories for the HWT scheme based on the following key criteria: being a laboratory of an institution with a formal WHO relationship, being a not-for-profit entity, being certified by the International Standards Organization (ISO), having a stable income of core activities, strictly adhering to harmonized test protocols/plans, agreeing to WHO ownership of test results and having confidentiality and freedom from conflict of interest measures in place. The aim is to designate laboratories in different geographic regions, with the status of designated laboratories being periodically re-assessed.

The WHO Scheme recognizes that, like other independent evaluation programs, WHO evaluation protocols must be living documents that are subject to periodic review and revision. The aim of the protocol is to guide designated testing laboratories, as well as other laboratories interested in testing according to WHO recommendations, in evaluating

HWT products in a scientifically rigorous, most efficient and cost-effective way possible. The latter is especially important to ensure that all products of public health relevance are evaluated and, in the future, to allow for more testing in low- and middle-income countries where the majority of products are distributed. The approach provides for modification or adjustment of the WHO testing protocols, thereby allowing the established procedures to be adapted to national policies and regulations as well as technological advances and new scientific discoveries. Approved modifications of the WHO test protocols for evaluating POU products utilizing different treatment technologies or combinations of technologies, provides for rigorous, yet adaptable, testing of the various types of POU systems.

The testing details of the EPA GS provided a detailed starting point for the creation of a harmonized test plan for the Scheme with the WHO Scheme Technical Officers, the IAC and other experts consulted on testing specifications. The selection of test water characteristics by technology type, test organisms for each microbial group, test capacity, sample points and the various operational considerations (i.e., conditioning, cleaning, component changes, end of life indicators, etc.) were deliberated and agreed upon. In some cases, further testing was done for informed decision-making prior to finalizing a harmonized testing protocol for the technology-specific test plans provided to the designated testing facilities. This facilitated their preparation of product-specific test plans for the technology performance evaluations. While acknowledging the rigor of the specifications of the US EPA GS and NSF P231 protocols that provided the basis for the similar WHO Scheme protocols, some departures of manufacturer-submitted data under these protocols have been considered for reduced testing under the Scheme. This so-called "Abbreviated Procedure" under the Scheme allows for data generated from locally accepted, relevant and recognized standards or protocols to be submitted to its IAC for recommendation to WHO for inclusion in the HWT Scheme, thus allowing for reduced Scheme testing. All submitted data must meet a level of independence, quality and sufficient rigor relative to the Scheme evaluation approach to allow for consideration.

In keeping with the commitment to maintain the WHO Scheme protocols as living documents, Round I evaluations (2014–2015) were according to the WHO Scheme Harmonized Protocol (2014). Round II evaluations (2016–2018) were according to a revised protocol, WHO Scheme Harmonized Protocol V2.0 (2016), which includes changes to the protocol to allow for efficiencies and improvements in the evaluation while retaining its rigor and robustness. Round III evaluations were according to the WHO Scheme Harmonized Protocol V3.0 (2019), which also reflects revisions from the previous V2.0. The Harmonized Protocol of the WHO Scheme that is largely derived from previously developed standards and guidelines will be further reviewed and analyzed in later sections of this document.

3. Adaption of Point-of-Use System Testing Protocols to Different Treatment Technologies, Water Qualities and Other Operational Conditions

Critical to the performance evaluation of each POU technology is a potential for its performance to be sensitive to or vary with the conditions for and levels of one or more water quality parameters which may significantly influence the ability of a POU system to eliminate enteric microorganisms (see Supplementary Table S2). In order to rigorously demonstrate that a POU device will maintain its ability to achieve specified microbe elimination targets in the course of both laboratory performance evaluations as well as in field or consumer use conditions, different types of POU devices need to be periodically challenged with representative and realistic influent waters of different compositions that include "worst case" qualities of water [24]. For example, POU devices that use filtration, adsorption, chemical additions, or UV radiation as the exclusive or primary means of microbial reduction can be compromised in performance by high turbidity (particle concentrations) in test water. High particle concentrations in water can physically shield adsorbed microbes from contact with disinfecting chemicals or UV radiation. Furthermore, the physical, chemical and biological nature of the turbidity particles can also influence the performance of the POU technology. This is because particles may exhibit differences in

oxidant demand, such as for halogens and other oxidant disinfectants, and have different radiation absorbing or scattering properties, such as for disinfecting UV radiation.

Elevated levels and certain sources of total organic carbon and high pH may also reduce the performance of some POU technologies. One such effect is to reduce the adsorption of viruses to ceramic, granular and other porous filter media. High concentrations of total organic carbon also can exert considerable oxidant demand, thereby neutralizing and chemically consuming much of the available oxidizing agent that is the basis of performance for oxidant disinfectants such as chlorine, chlorine dioxide, ozone and peroxides. POU systems utilizing halogen oxidizing agents or heavy metal disinfectants may be strongly affected by pH, as this parameter can change the chemical form of the disinfectant available and its antimicrobial potency. Consequently, POU devices should be challenged at intervals with microbe-containing influent waters possessing specific water quality characteristics that represent both typical and worst-case conditions.

Another consideration for challenge test influent water compositions is the potential for toxic or antimicrobial levels of residual disinfectants or other leached compounds to remain in the treated product water, without neutralization, sequestration or removal, after the prescribed contact time for the technology and before microbial assays. This continued exposure to the antimicrobial agent after the intended contact time or treatment process can result in further microbial reductions as a collateral effect not necessarily intended to occur and not necessarily accounted for in the design and conduct of the performance evaluation protocol. For example, antimicrobial metals such as silver can leach excessively from POU treatment systems into challenge water, especially for challenge waters with low pH and low levels of total dissolved solids (TDS).

In challenging a POU system that can leach antimicrobial agents into waters of certain composition, decisions need to be made in the design of the protocol about how to account for the possible presence and action of the antimicrobial agent in the treated water. Options are to neutralize, sequester or remove the leached chemical in the treated water being sampled or, alternatively, measure its concentration and account for its continued antimicrobial effects over time on the test microbes being analyzed. Analyzing the resulting treated product water for the concentration of residual or leached chemical also allows one to determine if the levels of this potential disinfectant exceed its recommended or allowable levels in drinking water.

In addition to specifying and utilizing distinct compositions of influent water to challenge different classes of POU technology devices, testing laboratories must also recognize that each POU design has its own unique characteristics (Supplementary Table S2). Each system may possess a different shelf- or use-life, water volume usage lifetime, flow rate and operating or use cycle. Certain systems may require component maintenance or replacement such as filter cleaning or replacement, UV lamp cleaning or replacement, or chemical disinfectant delivery unit replacement, either at scheduled time intervals, if flow rates decline, or if the disinfectant being delivered is exhausted or at a diminished concentration. The POU device should be set up and tested at the testing facility in such a way as to mimic its configuration and operation in the field to the extent possible. Manufacturers must provide operational parameters and maintenance information in documentation that accompanies each device. Laboratory testing staff must follow the manufacturer's instructions to ensure that the POU system is operated according to the manufacturer's specifications.

The measures taken to allow adaptability to different POU technologies, water qualities and other operational conditions for several standards and protocols are described below. The specific test water qualities and other conditions are also summarized briefly in Supplementary Table S1 for each of the protocols.

The US EPA Guide Standard and Protocol for Testing Microbiological Water Purifiers, published in 1987, explicitly stated that while it was written to address the testing of only three POU treatment technologies (halogenated resins, ultraviolet radiation and ceramic filtration candles), it was designed to be modified as needed to be used with changing

treatment technologies and advances in microbiology. The Guide Standard proposed that the protocol should be reviewed and updated every two or three years, although this review and updating schedule has not been met to our knowledge. While the EPA GS is used as a basis for other evaluation protocols, such as those of NSF and WHO, these decedents have incorporated the knowledge of improved or alternative microbial surrogates, assay methods, etc.

The US EPA protocol recommended a range of challenge water matrices to (1) evaluate the performance of different treatment technologies under realistic worst-case water quality conditions, and (2) to measure concentrations of potentially toxic disinfectant residues in the product water. The protocol advocates the practice that the manufacturer's operating instructions for each individual HWT system design must be followed during the entire testing process. However, microbial reduction performance requirements are identical for all types of treatment systems.

The NSF Protocol P231 for Microbiological Water Purifiers follows the principles and procedures recommended by the EPA protocol, while re-emphasizing that procedures should be modified for the testing of POU devices not specifically addressed by the EPA protocol, such as ultrafiltration technology, and should incorporate data-driven reasoning to allow for improvement in testing sensitivity, rigor, appropriateness or practicality in surrogates or methods, such as the use of bacteriophage MS2 over the human enteric viruses, Poliovirus and Rotavirus, for the evaluation of technologies that use size exclusion.

The NSF/ANSI 55 standard, Ultraviolet Microbiological Water Treatment Systems, was written specifically for the testing of devices utilizing ultraviolet radiation to eliminate microbes in drinking water. Initially developed in 1991, NSF/ANSI Standard 55's scope was limited to low-pressure ultraviolet (UV) radiation systems using low-pressure mercury bulbs, which was the only UV technology available in the marketplace at that time. In 2019, the scope was expanded to include light emitting diode (LED) based products.

This standard system was developed for two different types of water sources representing different water qualities. Class A point-of-entry (POE) and POU systems are designed to inactivate and/or remove microorganisms, including bacteria, viruses, Cryptosporidium oocysts and Giardia cysts, from contaminated water. Systems covered by this standard are not intended for the treatment of water that has obvious contamination from a known, intentional source, such as raw sewage, nor to convert wastewater to drinking water. The systems are intended to be installed on visually clear water that is not colored, cloudy, or turbid.

Class B point-of-entry and POU systems covered by this standard are designed for supplemental bactericidal treatment of disinfected public drinking water or other drinking water that has been tested and deemed acceptable for human consumption by the state or local health agency having jurisdiction. The system is designed to reduce normally occurring nonpathogenic nuisance microorganisms only. The Class B system is not intended for the disinfection of microbiologically unsafe water and may not make individual or general claims against protozoan cysts or oocysts or make microbiological health effect claims.

The Chinese document, Guideline for Declaration of Health Administration Permission for Products Relating to Health and Safety of Drinking Water (N. Ronnie, personal communication), provides minimal information for testing of different types of water treatment devices that are designed to remove chemical and microbiological contaminants, primarily by specifying quality parameters of the influent challenge water and maximum contaminant limits in the resulting product water.

The NSF Protocol P248, Military Operations Microbiological Water Purifiers (NSF 2012), which utilizes the EPA protocol and the NSF P231 protocol as the basis for its recommendations, was deliberately written to provide general guidelines which can be used to provide testing protocols for all types of POU water treatment devices. Like the US EPA protocol, the military protocol specifies two test water types, a general and a challenge test water phase. The general test water is identical for all technology (technology non-specific) and represents "unstressed" source water conditions. The Challenge Test Water

varies by technology to represent "stressed" source water conditions for that technology type to evaluate a realistic, worst-case, microbial reduction capability of POU devices utilizing different treatment technologies, including chemical disinfection, filtration and UV radiation. Consistent with the EPA GS, an additional test water is formulated to measure the leaching of potentially toxic silver into the product water yielded by POU systems incorporating silver as a disinfectant.

Water Quality India Association's WQIA)'s Guide Standard and Protocol for Microbiological Evaluation of Drinking Water Treatment Devices (known as WQIA Protocol IP 100) have been specifically developed for Indian conditions and match global norms [21].

None of the test protocols written by the Chinese Ministry of Health (2010), the Brazilian Association of Technical Norms (2004) or the Mexican Ministry of Health (2008) addressed the issue of adapting their respective protocols to alternate water treatment technologies.

Further details of the different protocols cited above and their testing requirements are provided in Supplementary Table S1 and in the text below.

4. Performance Standards, Guidelines and Testing Protocols for Microbial Reductions by POU Treatment of Drinking Water and Their Rationales

Several POU performance evaluation standards, guidance documents, recommendations and protocols are described below in terms of the required microbial reduction performance rationale. These documents are based on the programs and systems established and administered by WHO, NSF-International, US EPA and the governments of Brazil, People's Republic of China and Mexico. While not necessarily fully representative of and comprehensive for policies, procedures and practices used around the world, they represent the most recognized international sources of information as well as some example countries having their own systems.

4.1. WHO: Evaluating Household Water Treatment Options

The WHO recommends the use of local data on pathogen prevalence and human health risks in source waters in order to identify the target pathogens for technology performance evaluation, if such data are available or can be obtained [7,12]. Data on the presence, concentrations and documented health risks of the key or target pathogens identified should be used in Quantitative Microbial Risk Assessments (QMRAs) to derive locally relevant microbial reduction performance targets. However, in the absence of such local data, WHO recommends the use of default target pathogens and the needed microbial reduction performance targets for them to achieve levels of tolerable risk for microbial water quality. These microbial reduction performance targets are based on QMRAs for the default target pathogens specified in the WHO Guidelines for Drinking-water Quality [23] and their application to performance evaluation of POU/HWT technologies to meet tolerable health risk criteria [7,23].

As for the microbial reduction performance targets, WHO uses a tiered approach with corresponding tiers based on different levels of tolerable risk, expressed as DALYs (Disability Adjusted Life Years) [7,23]. Three microbial categories of target pathogens are specified in this approach: bacteria, viruses and protozoan parasites. There are three tiers of performance efficacy for each of these three different target microbes, each with specific $\log_{10}$ microbial reduction requirements. The "highly protective" tier, implemented as ★★★ performance by the Scheme, specifies that a water treatment technology reduces bacteria by 4-$\log_{10}$, viruses by 5-$\log_{10}$ and protozoa by 4-$\log_{10}$. The "protective" tier, ★★ performance under Scheme designation, requires the water treatment technology to reduce bacteria by 2 $\log_{10}$, viruses by 3 $\log_{10}$ and protozoan parasites by 2-$\log_{10}$. A third tier called "targeted protection", ★ performance under the Scheme, is provided for technologies that fulfill 2 of the 3 levels of $\log_{10}$ microbial reduction performance of the "protective" tier (★★).

The WHO approach and specifications for microbial reductions are derived from health-based targets for pathogens in water and their human health risks. This approach sets a desired health endpoint for acceptable risk level of illness and uses QMRA to determine the necessary level of microbial reduction by water treatment to achieve the health-based target level of tolerable risk. QMRA includes the key components of: (1) hazard characterization, (2) exposure and occurrence assessment, (3) health effects assessment, usually based on human infectivity and illness dose-response data and corresponding data fit functions (mathematical models) for specific pathogens, and (4) synthesis of exposure and health effects data to develop a characterization of microbial risks, typically for specific pathogens [7].

This characterized risk is described in DALYs, which is a common metric that quantifies the burden of disease caused by a pathogen that combines disease duration, severity of illness and extent of shortened life, in this case infection and illness resulting from waterborne pathogen exposure. The WHO uses this approach because it is a uniform metric that is used as a policy instrument to compare and prioritize multiple health risks of different kinds across an exposed population. WHO guidelines deem acceptable risk as 10^{-6} DALY per person per year, and based on this level of acceptable risk, the microbial reduction performance criteria were devised. "Highly protective"/★★★ technologies achieve this 10^{-6} DALY per person per year acceptable risk level, while "protective"/★★ technologies achieve a risk of 10^{-4} DALYs per person per year. A third "targeted protection"/★ level requires that 2 of the 3 microbial categories achieve microbial reductions and their corresponding DALYs per person per year consistent with "protective" performance. More detailed methods on how these performance levels were devised are described below.

In developing health-based targets using QMRA, reference pathogens were chosen to represent bacteria, viruses and protozoa. Reference pathogens chosen by the WHO are *Campylobacter jejuni* (bacteria), rotavirus (virus) and *Cryptosporidium* (protozoan parasite). These pathogens were chosen for their global presence in contaminated drinking waters, ability to cause disease, resistance to disinfection and for the abundance of information regarding their occurrence for exposure assessment and human infectivity dose-response relationships for health effects assessments.

Next, the expected concentrations of these representative pathogens present in untreated waters were estimated, assuming no waterborne outbreaks were occurring. The concentrations of pathogens in wastewater were first estimated using certain assumptions regarding pathogen concentrations in feces of an infected individual, percent of a population shedding pathogens in their feces, volume of sewage produced per person per day and natural inactivation of microbes in water. Then, an assumption was made that wastewater accounted for 0.01% of untreated water to result in pathogen concentrations for raw or untreated water.

Risk characterization calculations were then performed to describe the level of risk from pathogen presence in untreated waters. Using the estimated concentrations of each of the reference pathogens in untreated water, estimated daily pathogen exposures from drinking water were calculated assuming 1 L of water consumption per person per day. Estimated daily exposure was then converted to daily risk of infection, using each organism's dose-response function. Daily risk of infection was converted into annual risk of infection. Estimations for the probability of an infection developing into illness were then used to estimate yearly risk of illness per person. Finally, estimated DALYs per case of illness, along with the fraction of the population susceptible to each pathogen, were incorporated into the analysis and the disease burdens per person per year were calculated as DALYs.

From the risk characterization analysis, the necessary $\log_{10}$ reductions of the target pathogens of each type (bacteria, virus and protozoan parasite) to achieve certain risk levels were calculated. It was found that for treatment technologies to achieve the 10^{-6} DALY target risk level ("highly protective"/★★★), bacteria, protozoa and viruses must undergo 4, 4 and 5 $\log_{10}$ reductions, respectively. For the 10^{-4} DALY risk level ("protective"/★★), bacteria, protozoa and viruses must undergo 2, 2 and 3 $\log_{10}$ reductions, respectively. A

"targeted protection/★" technology needs only to achieve "protective" $\log_{10}$ reductions for 2 of the 3 microbe categories.

When locally available data for concentrations of the key target pathogens of each class in raw water are available, it is recommended that QMRAs be done to develop the $\log_{10}$ microbial reductions required by water treatment to achieve a target level of protection (highly protective, protective or minimally protective) based on DALYs per person per year, to develop local performance criteria. It is assumed that such QMRA analysis for risk characterization and the identification of the tolerable risk targets and corresponding $\log_{10}$ reductions are appropriate for health protection, based on existing conditions and contexts.

4.2. US EPA Guide Standard and Protocol for Testing Microbiological Purifiers

The US Environmental Protection Agency specifies testing POU treatment technologies against certain reference pathogens of bacteria, viruses and protozoan cysts. They also specify that POU technologies achieve a 6-log reduction of bacteria, 4-log reduction of viruses and 3-log reduction of protozoan cysts.

The EPA approach to specifying POU treatment microbial reduction performance requirements is based on assuming a worst-case scenario of high levels of microbial contamination in untreated water and setting POU technology performance levels as $\log_{10}$ reductions considered achievable by the POU technologies under consideration when the GS protocol was initially developed. For practical technology performance evaluation based on these $\log_{10}$ reduction targets, it was recommended that performance documentation be based on experimentally evaluating candidate POU technologies using test procedures and conditions specified and approved by EPA. Hence, the $\log_{10}$ microbial reductions targets were not established on the basis of formal and systematic QMRA, but rather on what was considered achievable by representative POU technologies available and widely used when the protocol was developed.

Reference pathogens chosen for POU testing include *Klebsiella terrigena* (now known as *Raoultella terrigena*) or *Escherichia coli* for bacteria, Poliovirus 1 and Rotavirus SA11 as test virus pathogens and *Giardia lamblia* or *Giardia muris* cysts as the protozoan reference pathogen. These reference organisms were chosen based on presence in contaminated waters, potential to infect and cause disease, extensive use in previous environmental studies, resistance to water treatment processes, differences in size and structure and susceptibility to treatment. Further details of the testing requirements and test conditions of the EPA GS Protocol for Testing Microbiological Water Purifiers are provided in Supplementary Table S1.

4.3. NSF P231: Microbiological Water Purifiers

The purpose of NSF Protocol 231 is "to establish minimum requirements for health and sanitation characteristics of microbiological water purifiers. The requirements are based on the recommendations of the US Environmental Protection Agency's Task Force Report, Guide Standard and Protocol for Testing Microbiological Water Purifiers (1987)". Therefore, NSF P231 is considered a reiteration and reinforcement of the EPA Guide Standard Protocol and does not require separate analysis in this literature review. Further details of the testing requirements and test conditions of the EPA and NSF Protocols for Testing Microbiological Water Purifiers are provided in Supplementary Table S1.

4.4. NSF P248: Military Operations Water Purifiers

NSF P248 applies to portable POU systems intended to purify natural waters of unknown microbiological quality. This protocol requires a 6-$\log_{10}$ reduction of bacteria based on using one of the specified bacteria surrogates, either *Escherichia coli*, *Raoultella terrigena,* or spores of *Bacillus atrophaeus*. Virus reductions of 4-$\log_{10}$ are required based on using both MS2 and fr coliphages (male-specific (F+) RNA bacteriophages of *E. coli*). Protozoan reductions of 3-$\log_{10}$ are required based on using Cryptosporidium parvum oocysts. The specified $\log_{10}$ reduction requirements were chosen such that an approved

device should remove or inactivate virtually any microbiological contaminant of health concern from any naturally occurring fresh water source. These log_{10} microbe reduction performance levels are consistent with the US EPA Guide Standard Protocol.

Some discussion on organism occurrence and surrogate selection was provided. The protocol's Appendix D Estimation of the bacterial concentrations in natural waters noted that coliform levels of 10^5 colony forming unit (cfu)/mL or higher are observed in typical polluted rivers and streams. Thus, a 6-log_{10} reduction of coliform group bacteria would be adequately protective for a typical polluted water source. This reasoning, however, was not explicitly stated within the protocol. For viruses, the protocol noted that observed virus levels in polluted streams are typically in the range of 10^2 enteric viruses per liter. The 4-log_{10} virus reduction performance target originally specified by US EPA in the Guide Standard Protocol and reflected here may have been established in part based on its realistic achievability with existing filtration technologies as well as resulting in reduced health risks. Cryptosporidium and Giardia (oo)cysts were observed in polluted waters up to around 10^2 (oo)cysts per liter, and it is likely that the specified 3-log_{10} reduction performance requirement is based on this level of occurrence as well as the evidence originally developed by US EPA GS for achievability with existing technology. If certain types of POU treatment technologies such as filters were to achieve a 4-log_{10} reduction of viruses and a 6-log_{10} reduction of bacteria, it is likely that the technology would also perform sufficiently well or better to achieve the specified 3-log_{10} reduction of the larger protozoan parasites, certainly for most filtration technologies.

However, assumptions about the achievability of log_{10} microbial reduction performance for one class of microbes based on performance for another class of microbes are not justified for different types of treatment technologies. This is because the basis for and mechanisms of microbe reduction can differ greatly among different classes of microbes according to the type of technology. Further details of the testing requirements and test conditions of the EPA Protocol for Testing Microbiological Water Purifiers are provided in Supplementary Table S1.

4.5. NSF/ANSI 55: Ultraviolet Microbiological Water Treatment Systems

NSF standard 55 addresses performance targets and standards for POU and POE ultraviolet systems. This protocol defines two classes of systems. Class A systems are defined as those that are intended to inactivate or remove microbes from contaminated water to a level considered to be safe. These systems are intended to treat water that may be microbiologically contaminated but are visually clear and relatively free from suspended particulates or other organics. Class B systems are intended for supplemental treatment of water that has been determined to be of acceptable quality and are not relevant to this literature review. Class A systems are relevant to typical POU technologies using ultraviolet radiation and will be considered here.

The microbiological performance requirements specified in NSF/ANSI 55 are intended to be consistent with the EPA Guide Standard performance requirements. Until 2019, rather than directly stating 6-, 4- and 3-log_{10} reduction requirements for bacteria, viruses and protozoan cysts respectively, there was instead a minimum UV dosage requirement specified. NSF 55 required that Class A systems deliver a minimum UV dose of 40,000 μW-s/cm^2 (equivalent to 40 mJ/cm^2) at a wavelength of 254 nanometers in buffered test water spiked with the indicator virus MS2 coliphage. This UV dose has been found to achieve a 4-log_{10} reduction of viruses and greater than a 6-log_{10} reduction of bacteria. The protocol also justified the 40 mJ/cm^2 dose requirement by noting that a UV dose of 10 mJ/cm^2 achieved 3- to 4-log reductions of *Cryptosporidium* and *Giardia*. Under this approach for Class A systems, the protocol required calibrating MS2 coliphage log_{10} reduction values against UV dose delivered. The log_{10} reduction value for MS2 at the minimum dose of 40 mJ/cm^2 then became the log_{10} reduction requirement for the POU technology's compliance with microbiological performance targets. The protocol stated that if this MS2 log_{10} reduction requirement is satisfied, the minimum UV dose is assumed to have been achieved.

NSF/ANSI protocol 55 only required the use of MS2 coliphage for Class A system testing. Choosing one test microbe instead of 3 or more was likely done for feasibility and cost savings on the part of the testing laboratory. By extrapolating from the observed MS2 $\log_{10}$ reduction to UV dose delivered, the protocol used past data on UV dose-response for other microorganisms to infer reductions of those other organisms that would be consistent with EPA Guide Standard performance targets. A virus surrogate was chosen over bacteria because of its greater resistance to UV disinfection, and over protozoan cysts due to their lower UV resistance, higher relative analytical costs and the specialized analytical capacities required for quantification of infectious (oo)cysts. Further details of the testing requirements and test conditions of the EPA Protocol for Testing Microbiological Water Purifiers are provided in Supplementary Table S1.

In 2014, the Drinking Water Treatment Units Joint Committee (JC) responsible NSF/ANSI Standard 55 began developing a protocol to address alternate UV technologies, expanding the scope to include UV-LED in 2019. Additionally, after investigations, the JC recommended a change in approach from the above-described indirect measure using MS2 to employing a direct log-reduction test with an organism, Q-beta, that would serve as a surrogate for all viruses. Q-beta, a male-specific (F+) RNA coliphage similar to MS2, was determined to be an acceptable surrogate for Rotavirus and the test method held true at both ends of the UV wavelength range examined (254–285 nm). Testing with Q-beta directly is also simpler, more consistent and less expensive than having to conduct the collimated beam study of the original approach.

The original protocol will remain in NSF/ANSI Standard 55 for at least 5 years. Manufacturers of 254 nm technologies will have the option to evaluate their product using the new or old test method defined in the standard. For devices other than 254 nm, the new method will be required with the following criteria:

- 4-log reduction of Q-beta at the alarm set point for Class A devices,
- 1.5-log-reduction of Q-beta for Class B with UV source irradiance at 70% normal output, or
- 2.14-log reduction with UV source irradiance at 100% normal output.

All changes to Standard 55 were ratified by NSF's Council of Public Health Consultants, which included representatives from US EPA and Centers for Disease Control and Prevention (CDC).

4.6. Brazil Standard NORM ABNT NBR 15176:2004

This protocol is relevant to gravity devices intended to "improve water quality", whether the water has already been treated for drinking or is raw source water. The protocol provides 3 separate categories for the classification of POU technology efficiency. A POU technology must satisfy at least one of the three test criteria of either (1) particle retention efficiency, (2) removal of free chlorine, or (3) bacterial $\log_{10}$ reduction efficiency, in order to be in compliance with this standard. Only the bacterial reduction efficiency requirement is directly relevant to microbial POU technologies, so only this requirement will be addressed here.

The bacteriological efficiency standard requires a POU device to exhibit at least a 2-log reduction of *E. coli* in water containing between 10^5 and 10^6 cfu/milliliter. The rationale for the bacteriological efficiency standard is unclear, other than stating that the device should improve bacteriological water quality. The 2-$\log_{10}$ *E. coli* reduction specification implies that this is sufficient reduction to qualify as improvement in water quality. Further details of the testing requirements and test conditions of the Brazilian requirements for POU testing are provided in Supplementary Table S1.

4.7. China Ministry of Health GB/T 5750-2006 Standard and Household and Similar Water Treatment Units (QB/T 4144-2019) Standard, Ministry of Industry and Information Technology

The Ministry of Health (MOH) standard of China has two bacteriological challenge requirements that must be satisfied. The first is the 1-log reduction of *E. coli*, resulting

in complete elimination (defined as zero cfu/100 mL) from distilled water containing 30–100 cfu/mL *E. coli* at quarter intervals of the device's estimated lifetime water volume capacity. The second requirement is to reduce total bacteria counts to <100 total plate count (TPC)/mL at the quarter intervals of the device's lifetime water volume capacity. If both of these requirements are satisfied, the device is said to be in compliance with the MOH standard. There is no specified requirement for reductions of viruses or protozoan parasites.

The MOH standard for microbial performance requirements has a large discrepancy between the required 1-log reduction of *E. coli*, and the allowable levels of *E. coli* post-treatment. It states that challenge water must have between 30 and 100 cfu/mL (equivalent to 3000–10,000 cfu/100 mL) but end with a final concentration of 0 cfu/100 mL. This reduction would equate to >4-$\log_{10}$ reduction, not the 1-log reduction specified in the protocol. Also, the rationale behind the specified TPC parameters in the finished water is unclear from the protocol. Further details of the testing requirements and test conditions of the China testing protocol are provided in Supplementary Table S1.

The China Ministry of Industry and Information Technology document of 2020 has a requirement to test for purifying microbial contamination, specifically total coliforms, in household and similar water treatments units. It requires test water to contain total coliforms at 200–2000 cfu/100 mL or most probable number (MPN)/100 mL as a geometric mean. If the water is treated to contain 0 total coliform/100 mL, the quantifiable $\log_{10}$ reductions would be 2.3 to 3.3 according to the microbial requirements of the document.

4.8. Mexico Standard PROY-NOM-244-SSA1-2008

This standard requires household treatment technologies to reduce mesophilic aerobic bacteria by 95% and reduce total coliforms by 99.99% (4-$\log_{10}$ reduction). It requires water from public supply systems to be spiked with aerobic mesophilic bacteria to 5000–10,000 cfu/mL and for coliform organisms to a concentration of at least 1600 cfu/100 milliliters.

This protocol states that its purpose is to provide standards for POU devices that help provide access to safe drinking water in areas of Mexico where public water supply may not be accessible or safe. The rationale behind specific microbial $\log_{10}$ reduction requirements is unclear. The protocol addresses only bacteria as pathogens of concern and does not mention viruses or protozoan cysts. Further details of the testing requirements and test conditions of the Mexico protocol are provided in Supplementary Table S1.

As indicated by the aforementioned information for specified test microbes, their expected or designated concentrations in water to be treated and the requirements for their reductions by POU treatment technologies, there are considerable differences in performance requirements among the different testing protocols and their corresponding standards. The considerable variability and inconsistency in the requirements, specifications or recommendations of these different POU performance evaluation protocols and performance requirements demonstrate the need for a more consistent and harmonized approach for specifying acceptable POU technology test microbes and performance targets. Many of the country-specific protocols are not health-based and are inadequate and inconsistent with current thinking. Even among the more prominent international protocols, there is different reasoning behind each of the specified choices of test microbes and their performance targets. The WHO protocol considers a health risk-based approach, whereas the EPA Guide Standard and resultant NSF-International protocols (P231, P248 and 55) favor a technical approach based initially on $\log_{10}$ reduction achievability assumptions for representative POU technologies and also applicability to "worst-case scenarios" of water quality and extended technology use. Several of the other protocols are silent on the link between performance measurements and their rationales for levels of performance. Overall, there appears to be no consistent or universal consensus on what POU technology performance requirements should actually be and how performance evaluation testing should be done. Greater efforts are needed in developing rational and practical consensus approaches, performance requirements and testing methods and materials internationally,

regionally and within countries to achieve harmonization and avoid confusion among various stakeholders.

5. Microbial Reduction Processes and Determining the Fate of Test Microbes Subjected to POU Treatment as Measured by Different Analytical Methods

POU treatment systems are designed to eliminate or reduce concentrations of pathogenic microorganisms from water by physical removal of the microbes or by exposure to disinfecting physical processes or chemicals that render the microbes noninfectious. Physical removal may be accomplished by various filtration processes which include porous ceramic filter elements (candles, pots and other configurations), granular filtration media, fiber-based depth filters or polymer-based membrane filters, including membrane filters with pore sizes small enough to retain viruses (ultra-, nano- and reverse osmosis filters) as the basis for the design of the POU device intended to sieve, trap, adsorb or otherwise physically retain microbes.

Chemical coagulation-flocculation and adsorption are other physical processes that can physically remove microbes from water and have been used for POU treatment. Both inorganic and organic coagulation-flocculation and adsorption technologies have been developed for POU treatment.

Physical removal can also occur as the result of biologically mediated processes (such as physical-chemical entrapment within or predation by cellular microbes and higher life forms) if a substrate-associated community composed of bacteria, fungi, protozoa, algae and other organisms is allowed to develop in a POU device. The development of such a biological community occurs as a biofilm on surfaces, in pores, or as a surficial layer in porous media, where it is referred to as a schmutzdecke. The intermittent flow, household scale slow sand filter, sometimes called the biosand filter, is an example of a POU system in which a schmutzdecke plays a major role in the retention and apparent biologically mediated reduction of pathogenic microorganisms.

Other POU treatments operate primarily by inactivating or killing microbes as they pass through the POU system into the product water or are added directly to the water without necessarily being physically removed. Disinfection, the processes by which microbes are rendered non-infectious or destroyed, may be achieved by exposure to chemicals that are strong oxidants (such as chlorine, bromine, iodine, chlorine dioxide and ozone) or other chemical agents such as silver, copper, brass and other antimicrobial metals that may render microbes non-infectious by slower and mechanistically different processes. Other chemical disinfectants, such as quaternary ammonium compounds, peracetic, acid and ferrates, are available to disinfect water, but they are not typically used for drinking water disinfection. Physical disinfectants include agents such as heat, UV radiation or exposure to sunlight within the treatment device. In some cases, the chemical agent may be released into the water as it flows through the device, thereby delivering the disinfectant to the water, and this residual of chemical disinfectant provides the necessary contact time to achieve microbial reductions.

Determining the extent of physical removal of microbes by a POU treatment device typically involves enumeration of the target microbes in both the POU influent (the challenge or raw water) and the product water (device or reactor effluent) using direct counting methods, often with the aid of a microscope to visualize the test microbes. This allows quantification of the differences in concentration between the influent and treated product water as a measurement of the extent of their reduction from the water. However, physical removal of enteric viruses or specific pathogen bacteria by a POU treatment system would be difficult to observe directly by visual means. This is because viruses require visualization by electron microscopy, which is a time-consuming and expensive process that requires high virus concentrations in the samples to be analyzed and does not lend itself to examination of a large sample volume.

For bacteria, if inoculated into the challenge water influent at much higher concentrations than any other bacteria likely to already be present in the challenge water or the POU system itself, one could obtain total direct counts of these bacteria in the influent challenge water and the treated effluent product water by direct microscopic analysis.

Often, such direct microscopic analysis is facilitated by direct staining of the cells with a fluorescent dye, such as acridine orange [24], and assuming that all of the observed bacteria were the specific challenge microbe type. However, it is often difficult, if not impossible, to detect and quantify the reductions of many specific bacterial pathogens because they are morphologically similar to the many other bacteria that may already be present in the water.

Direct microscopic counts of protozoan parasite (oo)cysts such as *Cryptosporidium* oocysts and *Giardia* cysts can be accomplished by staining with commercially available immunofluorescent antibodies for enumeration using a fluorescent microscope. The use of fluorescent latex or other polymeric microspheres with similar diameters and surface properties as *Cryptosporidium* oocysts in conjunction with fluorescent microscopy has been used as a less expensive, less biohazardous and more convenient nonpathogenic surrogate for evaluation of protozoan parasite removal by physical retention in POU treatment processes [25].

If physical removal was the only process utilized by a POU treatment system, the efficiency of microbe removal could be determined by enumerating the challenge microbes in the influent challenge water and the effluent product water using an appropriate method, possibly a direct counting technique, as described above. If, however, the POU system includes both physical removal and a disinfection process, it would be difficult to determine the contribution to treatment and efficiency of each individual process unless one can test each POU component or process separately. A viability or infectivity assay would be required to quantify the extent of microbe disinfection and this could be measured separately from the extent of microbe reduction due to physical removal from the challenge water, as determined by microscopic analysis.

5.1. Reductions of Enteric Viruses and Methods for Their Detection

POU systems can be challenged with human enteric virus pathogens like enteroviruses (such as poliovirus, echovirus or coxsackie virus strains), rotaviruses, feline calicivirus or murine norovirus (the latter two as a surrogate for human noroviruses). Some POU testing protocols specify the use of certain human enteric virus pathogens. All of the viruses mentioned above can be grown and enumerated using mammalian cell cultures. However, producing and maintaining mammalian cell culture requires a sophisticated and well-managed laboratory setting and trained staff. Because of the complexity, biohazard risks and higher costs of POU testing of culturable human enteric viruses, some POU protocols instead specify or recommend the use of bacteriophages of *E. coli*, called coliphages, as suitable indicators or surrogates for human enteric viruses. Certain coliphages have physical, chemical, morphological and other properties that are similar to those of many human enteric virus pathogens, and they can be easily, more quickly and less expensively propagated and enumerated using an *E. coli* or similar bacterial host. Hence, POU systems are now often tested for virus elimination or reduction using coliphage viruses as models for pathogenic human enteric viruses.

However, if a POU device is to be challenged with human enteric viruses which cannot yet be cultured readily or at all in the laboratory, like human noroviruses, certain infectious hepatitis viruses and certain enteric adenovirus strains, molecular-based detection methods utilizing polymerase chain reaction (PCR) methods for DNA viruses, reverse-transcription polymerase chain reaction (RT-PCR) methods for RNA viruses or quantitative PCR methods may be used. A major problem with these sensitive nucleic acid detection techniques is that the nucleic acid of a virus particle rendered noninfectious due to the effects of a deleterious environmental agent or a POU treatment process may still be detected, yielding a false-positive result. This is because a non-infectious virus or its released nucleic acid no longer poses a human health risk, and its detection by a nucleic acid-based amplification method is not predictive of a health risk [26]. This same drawback with nucleic acid detection methodology extends to the use of these methods for direct detection and quantification of bacteria [27] and protozoan pathogens [28] as well.

Various attempts have been made to minimize the problem of false-positive detection of non-infectious viruses by PCR and related nucleic acid amplification methods. An intact virus capsid not only protects the viral nucleic acid from degradation but is also essential for the critical step of virus adsorption to a specific receptor on the correct susceptible host cell prior to the entry of viral nucleic acid into the host cell, followed by viral replication. In a method designed as one step in a procedure to detect human enteric viruses from large volumes of water, a range of viruses with intact capsids were captured by adsorption to magnetic beads coated with pooled human serum immunoglobulins and then rinsed multiple times to remove free viral RNA and viruses with damaged capsids prior to RNA extraction and RT-PCR [29]. This process assumes that viruses which have been damaged by exposure to environmental conditions and agents to the extent that the viral capsids do not adsorb strongly to the immunoglobulins or that the viral RNA released from capsids are not indicative of human health risk. These virus remnants will be rinsed away, and the RNA from these degraded virus particles will not subsequently be amplified by RT-PCR. A method to minimize detection of viral nucleic acids, in this case DNA, from T4 coliphage viruses with heat-damaged capsids involved pretreatment of viruses with propidium monoazide prior to quantitative PCR [30]. Propidium monoazide is a dye that binds to DNA, preventing amplification by qPCR. The dye can penetrate the capsid only if the capsid is sufficiently damaged. Another method involves pretreating of RNA viruses exposed to various disinfecting processes with a combination of two enzymes, proteinase K and ribonuclease [31,32]. If viral capsids were sufficiently damaged during disinfection, the proteinase K enzyme could further digest the proteinaceous capsid, exposing the viral RNA to degradation by the ribonuclease enzyme, rendering the viral RNA non-amplifiable by qPCR or RT-PCR, thereby reducing false-positive detection of viruses considered to be no longer infectious.

The consensus opinion is that these enzymatic and chemical blocking methods of nucleic acids can reduce the incidence of false-positive detection from non-infectious viruses, but that the effectiveness of any of these procedures depends upon the presence of a certain degree of capsid damage, which varies with the nature, concentration of and exposure time to the disinfectant, as well as the type of challenge virus being exposed. Different viruses differ in their responses to different proteolytic enzymes, and there is no universal approach of enzymatic pre-treatment that provides consistent responses across the range of enteric viruses of interest. Consequently, the effectiveness of any false positive mitigation strategy would need to be validated for a given POU disinfection process and challenge virus. Furthermore, such pretreatment methods to minimize false-positive detections could not be used with chemicals or processes which damage only or primarily the viral nucleic acid while leaving the capsid intact and its virus receptors or attachment sites functional, such as UV radiation treatment.

5.2. Reductions of Bacteria and Methods for Their Detection

Most testing of POU technologies for bacteria reduction is based on the use of culture methods to quantify the bacteria in the challenge water and the POU-treated effluent water. As previously indicated, most POU testing protocols specify or recommend the use of a number of different fecal indicator bacteria rather than human pathogenic bacteria for such challenge testing. Some protocols are very specific as to the choice of test bacteria and the methods by which they are to be grown, prepared and cultured, while other protocols or recommendations are much less specific about the choice of test bacteria or their preparation and culture methods. Such variability and lack of specificity in the choice of test microbes and their preparation and assay methods also creates other potential sources of variability and uncertainty in POU testing methods.

Both pathogenic and indicator enteric bacteria, when exposed to sublethal stresses either in the environment or in a POU water treatment device, may suffer reversible damage to structural components of the bacterial cell or to metabolic processes. This damage can render the affected bacterial cell unable to replicate in the presence of toxic chemicals often

present in the selective culture media used for their quantification. These injured cells may, however, maintain their ability to grow in non-selective culture media or cause infections and disease in humans under certain conditions. These important phenomena of different and sometimes either reduced or increased detection of injured or stressed bacteria and the processes of bacterial resuscitation will be covered in later sections of this document.

5.3. Reductions of Protozoan Parasites and Methods for Detection

The oocyst stage of the enteric pathogen *Cryptosporidium parvum* is the most widely used challenge protozoan agent for POU treatment systems and other disinfection processes. A *Cryptosporidium* oocyst contains up to four infectious sporozoites which are released to infect epithelial cells of the intestine following ingestion by an appropriate host. One early technique to determine the viability of *C. parvum* oocysts was to perform an excystation assay. Following acid pretreatment, oocyst suspensions are incubated in a solution containing a bile salt and sodium bicarbonate, then observed microscopically to determine the proportion of oocysts exhibiting evidence of sporozoite excystation. Another method proposed for determining oocyst viability involved the simultaneous use of two fluorogenic stains, 4′,6-diamidino-2-phenylindoee (DAPI) and propidium iodide (PI). DAPI can pass through intact cell membranes of oocysts to bind to the sporozoite DNA, while PI, which also binds to nucleic acids, cannot pass through intact cell membranes, so it is excluded from live cells. Stained oocysts which exhibit fluorescence characteristic of DAPI but not PI are considered to be viable [33]. The excystation and dye inclusion/exclusion assay results were found to be highly correlated with one another, but both techniques greatly overestimated oocyst viability in comparison to infectivity assays in mice [34]. This overestimation of infectivity by viability staining and excystation has been well documented, especially for UV radiation disinfection. Infectivity assays using continuous mammalian cell culture lines have been demonstrated to yield comparable results to mouse infectivity assays for the detection of *Cryptosporidium* oocysts and their response to disinfection processes [35].

To avoid the considerable assay time, expense, training and infrastructure requirements associated with mammalian cell culture and live animal infectivity assays, attempts are being made to develop qPCR and RT-qPCR methods which detect infectious *C. parvum* oocysts while minimizing amplification of DNA or RNA from non-infectious oocysts [34]. For example, pretreatment of *C. parvum* oocysts with propidium monoazide, which can only enter cells with damaged cell membranes, reduces amplification of DNA from these presumably no-longer infectious oocysts by binding to DNA and blocking PCR amplification. Another approach is to amplify *C. parvum* oocyst messenger RNA, because mRNA is synthesized only by metabolically active cells and is degraded quickly if the cell is inactivated. A prominent mRNA target codes for the heat shock protein of *Cryptosporidium*, which is synthesized in response to cellular damage by deleterious environmental conditions. The ability of these modified nucleic acid amplification techniques to discriminate between infectious and non-infectious *Cryptosporidium* oocysts is, as with similar techniques designed to detect infectious enteric viruses, dependent upon the type and mode of action of the disinfection process as well as the conditions of exposure to the disinfectant.

The age of the challenge oocyst preparation is another variable in these assay methods, because *Cryptosporidium parvum* oocysts and their infectivity deteriorate relatively rapidly after recovery from infected animals and purification for use in experimental studies [36]. Although infectious *C. parvum* oocysts obtained from experimentally infected animals under controlled conditions are the recommended and default choice of test protozoan parasites for performance evaluation of POU technologies based on microbial inactivation or loss of infectivity, the use of surrogates such as fluorescent microspheres and even bacterial spores are considered potentially suitable substitutes to live infectious oocysts for performance evaluation of POU technologies based on physical removal treatment processes such as filtration and chemical coagulation. Given the limited availability and capacity to access and work with well-characterized strains of infectious *C. parvum* oocysts

in many locations globally, there is a need for further consideration of appropriate and practical protozoan parasite surrogates that can be more accessible and used more widely for performance evaluation of POU treatment technologies.

5.4. Evaluation Protocols and Microbe Viability Reduction or Physical Removal

At the time of publication, 1987, the US EPA Guide Standard indicated *Giaria lamblia* as the surrogate for the cyst challenge due to its widespread disease impact and resistance to chemical disinfection. The Guidance document did, however, foreshadow that new data and information may necessitate a review of the surrogate selection for cysts, naming *Cryptosporidium* specifically. Since the 1987 publication date, such research has been completed to support the use of Cryptosporidium. Already by the publish date (1987), the EPA Guide Standard referenced that the use of particles or beads for testing the occlusion filtration of cysts had been demonstrated (by NSF) to be an accurate and practical substitute for the use of live cysts and testing according to NSF-International Standard 53 would be acceptable for cyst. In fact, the only microbiological assay procedure which does not attempt to determine microbial infectivity or culturability is the alternative use of 4 to 6 µm microspheres, enumerated by direct microscopy, when testing for the physical removal of parasite cysts or oocysts by POU devices that utilize filtration alone to eliminate microbes from water. The NSF P231 protocol largely follow the US EPA recommendations, as does the NSF Military protocol P248. The mammalian cell culture infectivity assay is specified for use to quantify infectious *C. parvum* oocyst reductions, but fluorescent microspheres can be used to quantify reductions by POU technologies based on physical removal from water. Since the NSF/ANSI 55 Standard was written for ultraviolet radiation systems testing alone, only infectivity assays are appropriate for determining microbial reduction, and naturally chemical disinfectants would also require methods that evaluate inactivation.

In the Chinese testing protocol, Guideline for Declaration of Health Administration Permission for Products Relating to Health and Safety of Drinking Water, the only challenge microbes named are total and fecal coliform bacteria. No assay methods are explicitly named, but the implication is that standard bacteriology culture methods are to be used. Likewise, the Chinese Ministry of Health testing protocol, the Brazilian protocol and the Mexican protocol designated only bacteria as challenge microbes and recommended standard bacteriological culture methods for enumeration. However, the specifics of these standard bacteriological culture methods and their materials could differ among these protocols and be applied differently in the hands of different users.

6. Specification of Test Microbes

The choice of which test microbes to use for performance evaluation of POU/HWT technology is a critical issue that must address the wide range of pathogens potentially present in contaminated drinking water sources. This issue has already been considered to some extent in the earlier consideration of different protocols and standards used in POU/HWT technology performance evaluation. The different test microbes used for performance evaluation of POU/HWT technologies are listed in Supplementary Table S1. Here, the consideration of test microbe specifications for POU/HWT technology performance evaluation focuses on the basis for using surrogate, indicator or index microbes as representatives of waterborne pathogens. What to measure as evidence of microbial contamination of water has been a significant issue since municipal water systems, governments and others engaged in or concerned with the delivery of safe drinking water began providing water for public use. Water policies, practices and regulations have evolved slowly and progressively, but many microbiological parameters and methods for their analysis have remained the same for many generations. In terms of examining water for public consumption, the concept of examining water for evidence of fecal contamination associated with causative agents of infectious diseases first evolved from the seminal work of John Snow in 1854 after the outbreak of cholera in London from a public well contaminated with sewage [37]. Since this time, epidemiology has emerged as one of the major

disciplines used to study the transmission of infectious diseases through water after the outbreak has occurred [38]. As sanitary practices began to be established in the United States, the UK and Europe throughout the nineteenth and twentieth centuries, filtration and chlorination systems were increasingly used to disinfect drinking water for public consumption in order to prevent waterborne infectious disease [37].

Waterborne outbreaks have been recorded in the United States since the 1920s by various organizations, but in 1971, the Centers for Disease Control (CDC), the US EPA (United States Environmental Protection Agency) and the Council of State and Territorial Epidemiologists (CSTE) have kept collaborative surveillance records to monitor waterborne disease outbreaks [39]. However, it is generally agreed that the number of reported waterborne diseases are much less than those actually affecting the population as a result of the self-limitation of many of the diseases, access to adequate hydration in developed countries, attribution to other exposure sources and inadequacies in disease recognition, reporting and surveillance systems [40]. Nevertheless, the reported outbreaks provide representative information on which microbial pathogens are causing waterborne disease. Similar waterborne disease surveillance efforts that are also conducted in other countries around the world provide additional information on which pathogens are or can be present in fecally contaminated water and cause waterborne disease. For example, WHO Europe, along with UNICEF, has developed a guidance document on waterborne disease surveillance for the European region [41]. However, there is no uniform international harmonization of waterborne disease surveillance and reporting.

6.1. Fecal Indicators

Today, water utilities and other drinking water management authorities rely on an indicator organism approach to examine microbial quality of drinking water [37]. The use of fecal indicator bacteria to detect the possible presence of fecal pathogens in water has been an established practice in the drinking water field since the late 1800s [42]. The goal of the indicator organism approach is to detect the presence of human fecal contamination in a water source and to quantify, on the basis of indicator presence and concentration, the degree of contamination [37]. Though it may seem counterintuitive not to use the actual pathogens for the examination of fecal contamination, many such pathogens are present only at low and variable concentrations or are detected by only very complex, time-consuming and expensive processes [43]. The bacterial indicators utilized as evidence of fecal contamination in drinking water in most settings are coliforms or sub-groups of them that are easily detectable and quantifiable [37].

The rationale for the use of fecal indicators was described in the 1996 WHO Guidelines for Drinking Water Quality and in supporting WHO documents [44,45]. These documents emphasize the difficulty of assessing the risk posed by the presence of any of the many different pathogens in water and explain the difficulty in detecting and culturing these numerous pathogens, thereby resulting in the need for an indicator organism that is both easily detectable and culturable [46]. As a result of the need for indicator organisms, specific characteristics have been defined that relate to an ideal or preferred indicator. These characteristics are that the organism's persistence in water and its removal by water treatment are similar to that of waterborne pathogens, that the indicator organism does not grow in natural waters, that the organism is readily detected by simple methods and that the organism is always present in human feces in large quantities [47].

6.2. Fecal Coliform and Fecal Streptococci Bacteria as Indicators

Since the late 1800s, enteric bacteria shed in stools and found in fecally contaminated water samples, termed coliforms (and defined as Gram-negative, non-spore-forming facultative anaerobic bacilli that ferment lactose with the production of acid and gas within 48 h at 35 °C), have been a preferred fecal indicator bacteria group for water [48]. Despite this simple and specific definition, it is clear that there are multiple bacteria genera and species that meet the coliform classification but that may or may not be related to or exclusively

originate from fecal contamination [49]. Therefore, refinements have been made since the late 1800s to limit detection to those coliforms most specific to fecal contamination, with the newest tests designed to detect only *Escherichia coli*, the archetypical intestinal coliform.

In the WHO document Water Quality: Guidelines, Standards and Health, three groups of 'microbial indicators' are defined to eliminate ambiguity in the term. The three groups are: 'general (process) microbial indicators' that demonstrate the efficacy of a process, 'fecal indicators' that indicate the presence of fecal contamination and 'index and model organisms' that are indicative of pathogen presence and behavior [44].

As a result of various inconsistencies and discrepancies in the terminology and the growing evidence that some coliform bacteria are not of fecal origin but rather are environmental bacteria, modifications of the methods generally used for identification of coliforms were made to more specifically detect those bacteria of fecal origin. Specifically, a higher incubation temperature was utilized to prevent the growth and exclude the detection of the environmental coliforms and improve the fecal specificity of the indicator organism, with the intention to make them more representative of the bacteria present in the human gut [48]. Such tests are referred to as those for thermotolerant or fecal coliforms. However, other bacteria, such as *Klebsiella* spp. found in non-fecal environments as well as associated with fecal contamination, were found to meet the criteria for these 'thermotolerant' coliforms and were detectable by the methods used [50]. Hence, further efforts were made to find a more specific bacterial indicator that was related to human fecal contamination. Dufour et al. subsequently found that *Escherichia coli* is the preferred microbial indicator of fecal pollution as it is invariably found in the feces of warm-blooded animals [51]. WHO and other international and national authorities now recommend *E. coli* as the preferred fecal indicator of drinking water quality. As a result of the limitations and discrepancies that were discovered and the evolution of evidence for what constitutes a reliable fecal indicator organism, there have been additions to the specified test microbes as new or modified test protocols and standards were developed (see Supplementary Table S1).

Despite drawbacks like the potential to be environmentally sourced, fecally derived coliforms (identified as thermotolerant coliforms and *E. coli*) have been historically useful in identifying contaminated water. The main reasons for the use of these microorganisms are their ease of assay and decreased risk to the investigator. In general, it is recommended to examine water more frequently by means of a simple test than less often by a complex one [47]. However, many of these tests for fecal indicator analysis still require an extended amount of time (usually 24–48 h) before results are available, resulting in delays in identification and management of contaminated water.

While work on coliforms as fecal indicators was evolving over time, research was also conducted on Gram-positive coccoid bacteria known as fecal streptococci as important fecal pollution indicators [52]. The enumeration of fecal streptococci was further popularized and made more convenient when a selective medium was developed in Reference [53]. Fecal streptococci are characterized by high numbers in the feces of humans and warm-blooded animals, their common presence in wastewaters and fecally polluted waters, their absence from pristine waters and environments with no contact by humans or other animals, their environmental persistence and their supposed lack of multiplication in the environment [44]. Of the fecal streptococci group, the most specific indicators of human and possibly animal fecal contamination are the enterococci. There is evidence that enterococci survive for long periods in water and sometimes longer than *E. coli* and fecal coliforms [49]. Furthermore, enterococci are easy to quantify in contaminated water samples in 24 h with newer substrate-specific fluorogenic and chromogenic media [54]. Recent evidence suggests that enterococci are not only reliable indicators of gastrointestinal illness and other health risks from exposure to recreational water but also from exposures via drinking water [55]. While the merits of enterococci as fecal indicator and index organisms continues to be documented, there is no specific use of enterococci as test microbes for POU/HWT technology performance evaluation, as least for the protocols included in Supplementary Table S1.

6.3. Viral Indicators

As early as the 1940s and more conclusively in the early 1960s, it was found that enteric viruses such as polioviruses and the viruses responsible for infectious hepatitis (later shown to be hepatitis A virus and hepatitis E virus) were present in sewage and fecally contaminated water. Evidence for waterborne enteric viruses was based on detecting them in the water (polioviruses) and by epidemiological evidence from waterborne outbreaks (hepatitis A virus and E viruses), providing an additional level of concern for the microbial quality of drinking water [56]. With evidence that enteric viral contamination of water can also result from human fecal contamination, concerns were raised that fecal indicator bacteria may not be the most appropriate test microorganisms for addressing this virus risk.

As a result of waterborne viral illness risks, a number of studies were conducted such as those in Reference [57], in which it was concluded that coliforms were an inadequate fecal indicator parameter to assess viral contamination of treated drinking water. Due to this lack of association between coliform bacteria and enteric viruses, an alternative to coliform analysis was sought to analyze contaminated drinking water for viruses. As suggested by Kott [58], Grabow [59], Havelaar et al. [60] and Skraber et al. [61], one of the most appropriate models and indicators for viral contaminants in water are bacteriophages associated with fecal contamination, especially somatic and male-specific coliphages, due to their similar size and structural characteristics to human pathogenic viruses. The use of coliphages and other bacteriophages to detect fecal contamination of water goes back to the 1930s and 1940s. Despite their long recognition and use, only a small number of regulatory agencies have water quality criteria and standards for the regulation of bacteriophage indicators of fecal contamination in water samples. However, the US EPA has identified both somatic and male-specific coliphages as suitable indicators of viral contamination of groundwater [62] and the European Union identified somatic coliphages as a suitable drinking water quality virus indicator [63]. Existing POU evaluation protocols and recommendations specify the use of either human enteric viruses (such as polioviruses and/or rotaviruses) or coliphages (such as MS2 and fr) in challenge tests to quantify POU technology performance [64]. Enteric viruses of humans and animals have the disadvantages of being more technically difficult, time-consuming, biohazardous and costly to detect and quantify compared to coliphages, which are easier, faster, less biohazardous and less costly to use in POU/HWT technology performance evaluation challenge tests. Some performance evaluation protocols now incorporate viral indicators as well as bacterial indicators, and these test microorganism options and requirements are presented in Supplementary Table S1 for the relevant protocols.

6.4. Indicators of Protozoan Parasites

Outbreaks of gastrointestinal illness have been related to contamination of drinking water with enteric protozoan parasites as well [65]. Initial concerns were about waterborne amoebic dysentery from *Entamoeba histolytica*. However, since the 1960s, evidence emerged that the enteric flagellate protozoan pathogens *Giardia* sp. could be waterborne, and in the 1980s, it became clear that the coccidian protozoan *Cryptosporidium* spp. were also waterborne pathogens. *Giardia* sp. and *Cryptosporidium* sp. have accounted for the majority of reported waterborne outbreaks due to protozoan parasites. Again, evidence indicated that the coliform indicator group of bacteria was not able to adequately predict and identify protozoan parasite contamination of drinking water sources and supplies. In some cases, the outbreaks of protozoan waterborne disease occurred without any indication from coliform testing that the drinking water was fecally contaminated [66]. In general, protozoan parasites are more robust microorganisms than enteric bacteria, being resistant especially to chlorine disinfection. However, they are amenable to treatment by filtration as a result of their larger size compared to bacteria and viruses [48]. As a result of the limitations of coliforms and *E. coli* as protozoan parasite indicators for water quality management, other surrogate organisms have been suggested as fecal indicator organisms for protozoan parasites. Suggested bacteria include *Clostridium perfringens* and

other sulfite-reducing clostridia, the spores of which are resistant to disinfection [67]. In addition, spores of aerobic *Bacillus* species have also been proposed as a water quality and treatment indicator, even though these bacteria are not feces-specific [67]. Because the spores are relatively resistant to treatment, they have been used in some studies on treatment performance efficacy as protozoan parasite surrogates [68,69]. In addition, *Bacillus* spores have become a preferred test microbe for performance evaluation and validation of the efficacy of UV disinfection water treatment technologies [70].

Despite the potential to use surrogate microorganisms like the spore-forming bacteria *C. perfringens* and *Bacillus* sp. as model test microbes for protozoan parasite response to POU treatment, the most direct and conservative testing methods would be to examine the effect of treatment on the protozoan pathogens themselves. However, there are a number of drawbacks to these methods, including the need for specialized laboratory equipment and costly reagents, trained analysts and analyst proficiency testing with positive controls to verify that analytical performance is in the target range, poor method sensitivity in some test waters, potentially different and unverified detection methods used in different locations with different performance evaluation protocols and slow turnaround time for results, especially for infectivity assays [48]. Direct pathogen testing is a much-debated issue, but some water suppliers have already initiated some forms of direct pathogen testing [71]. Some current protocols specify the use of *Cryptosporidium* oocysts for POU technology performance evaluations, although the use of fluorescent microspheres as surrogate particles can be employed for testing technologies based on physical removal, such as various filter technologies.

Molecular technologies may make direct pathogen testing an achievable option in the future. However, indicator organisms and more resistant surrogates are currently the most convenient, simplest, effective and cost-effective options to evaluate the performance of drinking water POU technologies. The use of indicator organisms is not meant to be a substitute for pathogen testing in some contexts and circumstances, but it is a more accessible and basic testing approach that allows testing entities to conveniently, quickly and efficiently examine drinking water quality, determine its safety for public use and evaluate treatment technology performance. The European Union Drinking Water Directive has identified *Clostridium perfringens* and its spores as a suitable indicator for protozoan parasites in drinking water [63]. Hence, some test protocols reviewed in this document specify the use of indicator microbes rather than pathogens for protozoan parasite and other microbial technology performance evaluation.

7. Variation among Microbial Classes, Types, Species and Strains in Response to Point-of-Use Treatment Technologies

The purpose of this section is to determine if and to what extent there is variability and differences in microbial response to water treatment processes, and whether or how such microbial difference and variability is adequately recognized and addressed in the testing protocols for POU/HWT technology performance evaluation.

There are two general categories of accepted water treatment processes to reduce the presence or viability of pathogenic microorganisms in drinking water: disinfection (which refers to inactivation or killing of the microbe but often leaving it in the water) and physical removal of the microbe from water [72–74]. There are different processes and mechanisms by which the presence and risks of pathogenic microbes in water are reduced by these two main treatment categories. These differences impact both the choices of test microbes and the specific protocols for testing of particular water treatment technologies.

In microbial testing for performance evaluation of a POU/HWT technology, the more rigorous and thorough testing protocols specify the use of the three general categories of microbes, namely vegetative bacteria and/or bacterial spores, viruses and protozoan parasites or their surrogates. Such testing is done to determine how well each of these three types of microbes are physically removed and/or killed (or inactivated) by a specific POU/HWT treatment process or system. An important consideration is the choice of specific test microbes within each of the three categories. There are many species, strains and

sources, including environmental mixtures and isolates or well-characterized, archived and lab-grown strains or types of bacteria, viruses and protozoa, each with different physical, chemical and biological properties that influence treatment resistance or susceptibility. Another potential consideration regarding the response of the test microbes to the treatment process or technology is the effect it may have on the expression of the virulence properties of pathogens, an effect that may not be possible to directly model or quantify when using non-pathogenic indicator or surrogate test microbes. Addressing the expression of virulence of test microbes is not directly addressed by typical culture-based microbial assays and requires additional analytical methods. Because there are so many different potential pathogens in drinking water, it is not feasible to test a POU/HWT technology product on every species and strain of pathogenic bacteria, viruses, or protozoa. Therefore, certain organisms are chosen to act as representatives or surrogate indicators of that microbe class, and they are considered adequate to represent all the types of that microbe class for POU/HWT technology performance evaluation. Some protocols for POU/HWT technology performance evaluation have specified the use of indicator organisms, while others have specified the use of actual pathogens, especially for viruses and protozoan parasites (See Supplementary Table S1).

There is evidence that the ability of indicators to represent pathogens may not always be a valid assumption. There is considerable interspecies and even inter-strain variability in how sensitive microbes are to different types of drinking water disinfection and other treatment technologies [75]. Such microbial variability in response or susceptibility to specific treatment technologies may cause the results of the POU/HWT technology performance testing to be non-representative and even misleading, and performance of the technology to be incorrectly or incompletely characterized. The actual field performance of a POU/HWT water treatment technology in reducing or eliminating the many different types of waterborne pathogens present could be less effective and less predictable than results from laboratory testing with a few specific microorganisms would indicate. For example, a microbe used in testing chlorine disinfection may be relatively resistant to chlorine disinfection and thus serve as a conservative surrogate for chlorination technologies. However, such a microorganism may be less representative of the response of certain pathogens known to be relatively resistant to an alternative POU/HWT technology, such as susceptibility to UV disinfection in comparison to other test organisms that might be more representative of pathogen resistance to UV radiation. The assumption that a single indicator microbe is a conservative surrogate or indicator representative of different types of pathogens across all types of POU/HWT technologies may be incorrect and misleading. This may mean, for example, that a UV disinfection technology may require testing of a different surrogate microorganism than a chlorine disinfection technology in order to adequately represent the response to treatment of the more resistant but representative pathogen for each class of concern.

Much of the evidence regarding variability between microbe types, species, strains and sources is based on studies examining the effect of a specific treatment process on multiple microbial types, species, strains or sources, and observing the extent of variation of the effectiveness of that treatment process. This evidence is considered here by examining the observed variability in relative resistance to specific POU/HWT treatment processes of representative pathogenic or surrogate microorganisms for each microbe category, species, strain, or source, and the relationships among the three major categories of microbes in their resistance to different types of POU/HWT processes. Relative resistance of the microbial categories to chemical disinfection in general is, in order from greatest to least resistance: protozoan (oo)cysts and bacterial spores > viruses > vegetative bacteria [75,76]. This has been confirmed through numerous studies, though the degree to which the microbes are resistant differs with treatment type [76,77]. For example, the difference in resistance to disinfection between bacterial spores and enteric bacteria was found to be 400-fold for chlorine disinfection in one study, but only nine-fold for UV disinfection in another study [76,78]. Viruses, adenoviruses in particular, are known to be very resistant to UV

disinfection compared to the other microbe classes of concern, with both vegetative bacteria and protozoan parasites being less resistant [74].

Variation in microbe removal by filtration is considered a function of physical size as well as the adsorption and aggregation properties of the microorganisms [79]. Ignoring adsorption properties and electrostatic forces, the nature of filtration is dependent on size exclusion, and it is generally the case that filtration effectiveness, from most to least effective, is: protozoa (typical size range: 1–300 μm) > bacteria (typical size range: 1–10 μm) > viruses (typical size range: 0.02–0.3 μm) [80,81]. When adsorption is considered as a factor in the filtration process, for example, the use of activated carbon or positively charged ceramic or fibrous particle filter matrices to attract and adsorb negatively charged microbes, differences in filtration effectiveness among microbes of different sizes and surface properties may occur that are not solely dependent on size but also on surface charge and hydrophobicity [68,79].

7.1. Variation among Viruses in Response to POU Water Treatment

The effects of differences in the physical-chemical properties on microbes of the same general type and taxonomy on their responses to water treatment processes is documented based on empirical evidence. For example, viruses have differences in their surface proteins and other macromolecules (carbohydrates and lipids) that are often distinguished on the basis of antigenic types or strains and can be categorized by serotypes using antibodies. Therefore, differences in the responses of enteric viruses belonging to different taxonomic groups to physical and chemical disinfectants is expected and have been observed.

Enteric viruses recognized as health concerns in water and for which information on treatment efficacy is needed and available for some of them include: adenoviruses, enteroviruses (polioviruses, coxsackieviruses, echoviruses and others), hepatitis A and E viruses, noroviruses, reoviruses, rotaviruses and indicator viruses (primarily bacteriophages (coliphages) of *E. coli* such as male-specific (F+) coliphages (e.g., MS2, f2, Q-Beta, fd, fr and others)) and somatic coliphages (Microviridae such as PhiX-174, Tectiviridae such as PRD-1, Myoviridae such as T4, Siphoviridae such as T5 and Podoviridae such as T7) [59,82]. For example, such taxonomic differences among enteric viruses are documented for differences in responses to water treatment processes such as disinfection with free chlorine and with UV radiation [75,82–84].

While studies have examined the physical removal or disinfection of many of these viruses, the choice of which of the enteric viruses to use for POU/HWT technology performance evaluation has not been made on a clear and consistent basis, and different viruses are specified or recommended in different protocols. A comparison of the responses of some different pathogenic viruses and indicator viruses to disinfection and to physical removal processes is described below for some common types of POU/HWT technologies.

Chlorination is often used for POU water treatment and is the most widely recommended chemical disinfectant for drinking water. There is evidence of considerable variability in virus response to chlorine among virus taxonomic groups and strains. A study in Reference [85] examined the effect of chlorination on 25 enteric virus strains in surface water, and found considerable variability in the Ct values required to achieve 99.99% reductions with 0.5 mg/L free chlorine dose [86]. Reovirus 1 required only 2.7 min to achieve this reduction, whereas coxsackievirus A6 was predicted to require 43.7 min. Observed contact time to achieve 99.99% reduction of coxsackievirus was found to be over 120 min, likely due to aggregation of the virus which may have provided additional protection from disinfection to the inner virions within the aggregates.

Another study examined the relative resistance to chlorine of several poliovirus and coxsackievirus isolates from environmental sources and drinking water at a 0.4 mg/L free chlorine dose [87]. Variation among isolates of each virus and between the two virus strains was observed. Coxsackievirus B5 isolates showed as much as 70% survival after 10 min of contact time, whereas the poliovirus isolates had less than 0.9% survival, with many at <0.003% survival after 10 min of contact time. Another study comparing inactivation of

viruses by chlorine (at 0.5 mg/L) found there to be a wide range of inter-strain variability in susceptibility to disinfection [83]. It was also concluded that differing conditions of the treated water (such as pH) could change the level of inter-strain variability and alter relative susceptibility of the viruses to disinfection by chlorination.

Differences in response to water treatment can also change according to treatment type. One study compared the relative resistance of f2 coliphage, human rotavirus and rotavirus SA11 strain to disinfection by chlorine (2 mg/L chlorine) and by ozone (0.26 mg/L) [88]. The study found that f2 coliphage was one of the most resistant viruses to chlorine but was very susceptible to ozone, and human rotavirus was much more resistant than the simian SA11 strain to chlorine and ozone treatment. A study examining ozone disinfection of poliovirus type 3 and MS2 coliphage in phosphate-buffered water found that for MS2, there was a mean of 1.6-log units more inactivation from ozone than poliovirus 3 under the experimental conditions tested (ozone concentrations of 0.60, 1.29 and 1.76 mg/L) [89].

UV irradiation (whether artificial or from sunlight) also elicits considerable differences in viral susceptibility to disinfection. A study examining UV irradiation on adenovirus type 2, echovirus 1 and 2, coxsackievirus B3 and B5 and poliovirus 1 found that adenovirus (AD) type 2 needed 4–6 times the dose of the other enteric viruses to achieve 99% [90]. Another study of UV disinfection comparing adenovirus serotypes, MS2 and poliovirus 1 found that adenovirus strains showed slight variability, and most strains showed similar resistance to UV disinfection as MS2 [91]. However, one strain, AD40, showed higher UV resistance than the other viruses tested. Poliovirus was more easily inactivated than the other tested viruses.

Filtration technologies that are widely used for POU water treatment have also given differences in the extent of virus reduction for the different viruses tested. Evidence suggests that observed differences in virus reductions by filtration are due to size or structural differences, surface charge (related to isoelectric point), surface hydrophobicity or adsorption or aggregation tendencies among the viruses [78]. In certain technologies using positively or negatively charged surfaces to enhance physical removal, the choice of virus can greatly affect results, as negatively and positively charged viruses will respond differently in the extent of adsorption when encountering charged surfaces. In a study of the adsorption of poliovirus 1, reovirus types 1 and 3 and coliphages MS2 and T2 to colloidal silica synthetically modified to carry either positive or negative surface charge, all viruses adsorbed exclusively to negatively charged silica at pH values below their isoelectric point, where they had positive surface charges, and all of them adsorbed exclusively to positively charged silica at pH values above their isoelectric points where they had negative surface charges [92]. There is also evidence that certain viruses are more likely to aggregate than others, which can change filtration efficacy. A study examining ceramic filtration of viruses found no significant removal of bacteriophages MS2 and PRD1 (small and spherical phages), but saw 2–3-$\log_{10}$ removal more of the bacteriophage of the family *Siphoviridae* (comparatively large, tailed bacteriophages) [80]. It was postulated that the observed differences in reduction were due to the physical-chemical properties of the larger, tailed bacteriophage of the family *Siphoviridae* that likely experienced hydrophobic interactions, which may have encouraged aggregation (and thus become easier to filter out), compared to the smaller, spherical and perhaps more hydrophilic bacteriophages tested.

Coagulation is a widely used form of physical-chemical removal for larger scale water treatment systems that has relevance for certain POU household treatment systems because of the use of coagulants such as alum, ferric salts and extracts from moringa seeds to coagulate household water [5]. Coagulation can be highly effective in removal of viruses, but the extent of virus removal can be variable among different viruses, coagulants and water qualities [93–95]. The extent to which there are differences among viruses during coagulation has been studied but remains inadequately characterized, especially under field conditions. In lab studies on the removal of poliovirus type 1 and coxsackievirus A2 from water and biologically treated sewage effluents, virus reductions by coagulation with iron and alum salts ranged from 1 to 5 $\log_{10}$ [94]. A study examining virus removal

by coagulation with ferric chloride found some differences in virus removal (0–1.0-$\log_{10}$ differences) among MS2, fr, PRD1 and phi-X174 bacteriophages [96]. Another study found little difference in physical removal between bacteriophages Q-beta and MS2 using aluminum salt coagulation [97]. The extent to which the reductions of bacteriophages by coagulation as a POU/HWT process is predictive of the reductions of human enteric viruses remains uncertain, especially under field conditions.

7.2. Variation among Bacteria

Differences in the responses of different bacteria to water treatment processes have also been observed and can differ according to species and strain of bacteria, as well as by type of water treatment. The extent of bacteria reduction by water treatment processes has been reviewed by the authors of References [73,74], who reported great variations in bacteria, as well as virus and protozoan parasite reductions by different water treatment processes in water of different quality and with different water treatment operating conditions. Different bacteria had different reductions by particular water treatment processes, and for specific water treatment processes, different bacteria had different reductions as well. Such differences in bacteria reductions by type of bacterium, type of treatment process and under different water quality and operating conditions make it challenging to determine which test bacteria to use for POU/HWT technology performance evaluation.

The different performance evaluation technology protocols and standards highlighted in this report specify the use of just a few different test bacteria, with the Gram-negative coliform bacteria such as *Raoultella terrigena* and *E. coli* being the ones most widely used, followed by *Bacillus* sp. spores. The extent to which these specified test bacteria adequately represent the microbial reductions by treatment processes of other waterborne bacteria of health concern remains uncertain. It is widely assumed that the coliform bacteria specified in POU/HWT testing protocols, such as *R. terrigena* and *E. coli*, will give reductions by water treatment processes that are similar to those for Gram-negative enteric bacteria pathogens that are taxonomically and morphologically similar, such as *Salmonella* sp., *Shigella* sp., *Campylobacter* sp. and *Vibrio* sp.

Some waterborne bacteria of human health concern that are taxonomically and morphologically different than either the Gram-negative rod-shaped bacteria or the Bacillus spores used in test protocols for POU/HWT technology performance evaluation may show different responses to water treatment processes. *Mycobacterium avium*, for example, had different responses to different water treatment technologies. They are very resistant to chlorination and ozone disinfection but are variable in their resistance to UV irradiation. They are not as sensitive to UV radiation as Gram-negative bacteria such as *E. coli* [98,99]. Mycobacteria species, however, show interspecies variability in response to disinfection processes. A 2002 EPA study found that *Mycobacterium fortuitum* required a UV dose of 7 mJ/cm^2 to achieve a 90% reduction, whereas a dose of 66 mJ/cm^2 was required to achieve a 90% reduction of *Mycobacterium intracellulare* [100]. *M. avium* cells in water tend to aggregate or adhere to larger particles, which has the effect of causing them to be removed well by coagulation, sedimentation and filtration, but to have increased resistance to chemical disinfection and UV irradiation [77,101].

Certain enteric bacterial pathogens have shown differing resistance to disinfection by chlorine. For example, one study found that *Campylobacter jejuni* was more susceptible to both chlorine and monochloramine treatment than *E. coli* [102]. Differences in susceptibility to UV or SODIS have also been found among several enteric bacterial pathogens. In a study comparing bacterial survival under sunlight in river water, it was found that *Campylobacter* was inactivated at approximately 10 times the rate as *E. coli* and *Salmonella* [103]. Another study examining SODIS found that it took 20, 90 and 150 min to achieve at least a 4-$\log_{10}$ reduction of *Campylobacter jejuni, Escherichia coli* and *Yersinia enterocolitica*, respectively [104].

Some enteric bacteria have shown considerable variability even between strains in response to free chlorine disinfection. For example, *Aeromonas hydrophila* strain TW11 was found to be 7 times as resistant to chlorination as *A. hydrophila* strain TW27, with Ct99

values of 1.4 and 0.2 mg-min/L, respectively [105]. *Mycobacterium avium* bacterial strains were found to have free chlorine residual Ct99 values ranging from 51 mg-min/L for *M. avium* strain 5502 to 204 mg-min/L for *M. avium* strain 1060 [101].

However, other strains of the same species of bacteria have shown similar resistance to disinfection. *Mycobacterium avium* strains 5002 and 5502 were found to have similar resistance to ozone disinfection, with Ct99 values of 0.12 and 0.10 mg-min/L ozone, respectively [101]. Another study found that many vegetative bacteria were similar in UV disinfection resistance [76]. The UV dose to achieve a 99.9% reduction was approximately the same (about 7 mJ/cm^2) for *E. coli*, *Staphylococcus aureus*, *Shigella sonnei* and *Salmonella typhi*. However, *Streptococcus faecalis* required a 1.5 times higher UV dose (about 10 mJ/cm^2).

Because of similarities in their physical size, it would be expected that most bacteria would respond similarly to physical removal by filtration processes that involve size exclusion and mechanical straining. However, some bacteria have also shown differences in response to water treatment by certain granular media filtration processes. A study examining slow sand filtration found about a 1-log$_{10}$ difference in removal between *E. coli* and *Campylobacter pylori* (2.6-log$_{10}$ versus 3.4-log$_{10}$, respectively) [106].

7.3. Variation among Protozoan Parasites

Protozoa also exhibit differences by genus, species and strains in response to disinfection and physical removal water treatment processes. A review study by the EPA on waterborne pathogens found considerable differences in chlorine inactivation of amoebic protozoans *Acanthamoeba* and *Encephalitozoon* species, with Ct99 values of 1000–7000 and 36–114 mg*min/L, respectively [77]. Another study examined SODIS effectiveness against a range of enteric parasites or their surrogates, and found considerable variability in susceptibility to SODIS [107]. Using 550 W/m^2 UV radiation intensity over 6 h, log$_{10}$ reductions in viability were: *A. castellanii* cysts = 2.16, *N. gruberi* cysts = 3.59, *E. invadens* cysts = 1.92, *G. lamblia* cysts = 1.96, *C. parvum* oocysts = 0.32 and *A. suum* ova = 1.42. Another study of SODIS disinfection found that after a total dose of 12 kJ UV/sunlight, *Giardia muris* cysts were completely noninfective, whereas a 30 kJ total dose was required to render *Cryptosporidium parvum* oocysts completely noninfective [108].

Effectiveness in physical removal of protozoa using filtration technologies is generally dependent on size. For example, a study found that 5 µm polystyrene latex spheres were removed within +10% of *Cryptosporidium parvum* oocysts of approximately equal size when examined in bench-scale filtration [109]. The results of these studies provide evidence that filtration effectiveness against parasites is largely dependent on microorganism size, with greater reductions of larger size organisms. Overall, studies document that microfilters and small pore size membrane filters extensively remove both *Cryptosporidium* and *Giardia* (oo)cysts from water [110].

Because testing water treatment technology performance with actual protozoans and other parasites is technically demanding, tedious, biohazardous and costly, it is often easier to measure the responses of bacterial spore indicators as surrogates for protozoan cysts or oocysts to quantify reduction efficacy by water treatment processes. However, there is evidence that bacterial spores may not always be appropriate, and that there are considerable differences between the bacterial spores and the protozoan (oo)cyst responses to treatment processes. One study examining UV disinfection technology performance found that *Bacillus subtilis* spores, which are often used as protozoan parasite surrogates, were about half as resistant to UV disinfection as *Acanthamoeba castellanii* cysts [76]. A study of chlorine dioxide disinfection found that *Cryptosporidium parvum* oocysts infectivity was reduced by 2-log$_{10}$ at variable rates for three different sources of oocysts, with Ct values of 75, 550 and 1000 mg*min/L. In contrast, the surrogates of *Clostridium* spores and *Bacillus* spores were inactivated by 2-log$_{10}$ units or more at Ct values of <50 mg*min/L [111]. Hence, different sources of infectious *C. parvum* oocysts can differ in magnitude of chemical disinfection efficacy by greater than an order of magnitude, and bacterial spores used

as possible *Cryptosporidium* oocysts surrogates can be more sensitive to certain chemical disinfectants than are *Cryptosporidium* oocysts.

7.4. Laboratory Strains of Microbes Compared with Environmental Isolates

Evidence documents that the sources or origins of test microbes can influence test microbe responses to water treatment technologies for a range of different reasons. There may be appreciable differences between laboratory grown microorganisms and environmental sources of the same kinds of microorganisms in their reductions by water treatment technologies. Responses by environmental isolates of pathogens to water treatment can be highly variable and unpredictable. For example, a study examining relative resistance to chlorine of isolates of polioviruses and coxsackie viruses from environmental sources and drinking water found that isolates from environmental sources and chlorinated tap water were more resistant to chlorine than were laboratory-maintained strains of the same virus type [87]. Another study found that poliovirus isolates from chlorinated tap water were more resistant to chlorine disinfection than laboratory grown strains of the same type [112]. A study examining sulfite-reducing clostridia disinfection by UV (254 nm) radiation found that environmentally isolated clostridia were 1.6 times as resistant than were lab-cultured spores [106]. Potential reasons for such differences between lab-grown strains and environmental isolates are addressed in another section of this literature review. A confounding issue in the use of environmental sources of test microbes (such as mixtures of them in sewage) is their heterogeneity. For example, UV and thermal disinfection studies of somatic coliphages have shown that they can differ greatly among the members of different somatic coliphage taxonomic groups, and that the occurrence of these different somatic coliphages in sewage may be variable and uncertain [113].

7.5. Test Microbe Selection by Point-of-Use Evaluation Protocols

Given the diversity and extent of differences in reductions of different microbes by different POU treatment technologies, there is a need for further consideration and evaluation of candidate test microbes for use in performance evaluations of different POU treatment technologies. Therefore, there is merit in further considering choices of test microbes based on local evidence of which pathogens are most relevant and appropriate as target microbes based on occurrence in water and magnitude of human health risk and which test microbes are most representative of their responses to candidate POU treatment technologies.

Below are listed certain protocol specifications on type(s) of reference organisms required or recommended for microbial disinfection and other treatment performance evaluations, and how well each addresses or accounts for microbial differences in response to POU/HWT technologies. It is noteworthy that the test microorganisms specified to represent a major class of microorganisms differ among the different protocols and standards. Some protocols and standards specify the use of actual pathogens, such as the enteric viruses poliovirus and rotavirus, and the protozoan parasites Giardia and Cryptosporidium, while others specify the use of coliphage indicator viruses, such as MS2 and fr, and will allow the use of fluorescent polystyrene spheres instead of protozoan parasite (oo)cysts.

7.5.1. WHO: Evaluating Household Water Treatment Options

The WHO performance evaluation document of 2011 [7] leaves room for choices and adjustments with respect to the selection of pathogen or reference (indicator) microorganisms for POU/HWT technology challenge testing. The document recommends that pathogens or surrogates be chosen on a case-by-case basis for a specific technology and geographic location. When possible, the document recommends testing enteric pathogens that are geographically relevant, have a high associated disease burden, are resistant to disinfection and are associated with and persist in fecally contaminated waters. Further information on the WHO recommendations may be found in Supplementary Table S1.

For bacteria, the WHO protocol recommends certain strains of *Campylobacter* (*C. jejuni* or less pathogenic strains), *Vibrio cholerae, Escherichia coli*, or certain *Salmonella* species as credible test candidates. *Campylobacter* sp. is an example of a reference bacterial pathogen in the WHO Guidelines for Drinking-water Quality (GDWQ) [23], which is why they are recommended here, if otherwise justifiable based on local conditions. When direct bacterial pathogen testing is not possible or appropriate, the document recommends testing non-pathogenic coliform bacteria such as non-pathogenic strains of *Escherichia coli* (e.g., *E. coli* strain B) or *Enterococcus* species (e.g., *E. faecalis*, *E. faecium* or other species).

The WHO document recommends rotavirus as a suitable viral test candidate because it is the reference viral pathogen of the GDWQ. Acceptable alternatives were identified as coliphage MS2, Echovirus 12 (an enterovirus), φX-174 (a coliphage), PRD1 (an enteric bacteriophage), or other bacteriophages. They are similar in structural morphology, composition and behavior to certain human enteric viruses, which provides a basis for their relevance and representativeness in evaluating the virus reduction performance of POU/HWT technologies.

For protozoan parasites, the preferred organisms for testing are *Cryptosporidium parvum*, *Giardia lamblia* and *Giardia muris*. *C. parvum* is recommended because it is the reference pathogen in the GDWQ. Acceptable alternatives are identified as *Clostridium perfringens* spores, *Bacillus* spp. spores or naturally occurring spore-forming bacteria as surrogate indicators, *Entamoeba histolytica* or *Entamoeba* spp. as alternative protozoan pathogens of possible health concern in some settings, or inert synthetic microspheres as particle surrogates for mechanical filtration treatment technologies that function on the basis of physical removal or size exclusion from water.

The WHO recommendations for bacterial, viral and protozoan challenge microbes for POU/HWT technology performance evaluation provide a wide array of choices, and encourage POU technology verification programs to choose the most appropriate microorganisms for testing based on context and capacities. However, the lack of specific requirements regarding choice of test organisms for specific POU technologies presents the opportunity for biased, advantageous or less representative selection of certain microorganism species or strains to characterize the performance of a specific POU treatment technology. For example, the use of *Campylobacter jejuni* (which is more sensitive to UV disinfection than *E. coli* or *Salmonella*) in testing a UV-based technology may give misleading and inadequately representative results, as it would not be the most conservative choice of bacteria for testing this type of technology. In this way, the WHO document recommendations for protocols' development perhaps does not sufficiently address microbial variation in selecting representative test microbes for POU/HWT technology performance evaluation.

The WHO document approach does emphasize the need to consider the pathogens with the highest regional prevalence and contribution to waterborne disease when making test microbe choices for POU/HWT technology performance evaluation or verification. This approach differs from many other documents that are specific protocols with specified test microbes because it allows for adaptability by taking into consideration different geographic locations and their conditions with respect to waterborne pathogen occurrence, disease risks and burdens. Therefore, despite certain drawbacks of not specifying exactly which microorganisms must be used in testing, the advantages of this approach likely make it a suitable guide for the location-specific development of POU/HWT technology performance evaluation or verification protocols.

As previously noted, however, the WHO has also addressed the need to harmonize and provide a consistent approach for technology producers and other stakeholders who seek to have the performance of a specific candidate technology tested in a way that can lead to broad recognition of its performance according to the WHO performance categories. Therefore, WHO developed a technology testing and performance evaluation mechanism called the WHO International Scheme to Evaluate Household Water Treatment Technologies (The Scheme). This Scheme establishes a mechanism for candidate commercial POU/HWT technologies to undergo a process of testing by a specific, prescriptive testing

protocol by a WHO-designated laboratory to produce microbial reduction performance data that can be reviewed and assessed by a WHO Independent Advisory Committee for recommendation for possible inclusion in a list of acceptable treatment technologies that meet WHO performance criteria. The Scheme uses a harmonized protocol and technology-specific protocols that detail the organisms and acceptable methods to be used for the evaluation, the test water characteristics and their preparation materials and methods of analysis for ensuring waters meet specifications, disinfectant neutralization methods, quality control criteria, operation of systems, testing capacity, etc.

In early 2016, the first global assessment of HWT performance was launched as Results of Round 1 of the WHO International Scheme to Evaluate Household Water Treatment Technologies in a report which included results of products evaluated under Round 1 (2014/2015) and results of a rapid market assessment of HWT in Africa and Asia (Ethiopia, Ghana and Vietnam). Round 2 evaluations of the WHO Scheme were published in December 2019 [114]. Round 3 was initiated during the drafting of the Round 2 report and was ongoing at the time of this manuscript preparation. Capacity building efforts in HWTS performance evaluation according to the WHO Scheme are in progress in a growing number of countries, including Ethiopia, Ghana and Vietnam.

7.5.2. EPA Guide Standard Protocol and NSF P231: Microbiological Water Purifiers

The EPA Guide Standard Protocol has specific requirements in the choice of test microbes for POU/HWT technology performance evaluation or verification. Reference pathogens specified for POU testing are *Klebsiella terrigena* (now known as *Raoultella terrigena*) for bacteria, poliovirus 1 and rotavirus SA11 (a simian rotavirus) as test viruses and *Giardia lamblia* or *Giardia muris* cysts as test protozoan reference pathogen. These reference organisms were chosen based on presence in contaminated waters, potential to infect and cause disease, extensive use in environmental studies, resistance to various types of water treatment processes, differences in structure and response to treatment (among microbial categories and among their types and strains).

R. terrigena was chosen as the bacterial organism because it is a common coliform that is often found in surface waters, and its presence has been shown in the past to be strongly associated with other enteric pathogens in waters. The Guide Standard Protocol only requires this bacterium to be tested, perhaps discounting bacterial variability by justifying that viruses and protozoa must also be tested and presuming that viruses and protozoa will be more resistant to disinfection, and viruses will be less removed by filtration than bacteria. However, this protocol does not account for the possibility of bacterial mutation, horizontal gene transfer or other genetic or physiological differences that could lead to greater bacterial resistance to different types of disinfection or other treatment processes, such as increased resistance to antimicrobial metals or chlorine, presence of cell surface external layers of polysaccharides (capsules or slime layers) or properties causing greater ability to penetrate a filter medium.

Poliovirus and rotavirus were chosen at the time for their high occurrence in fecally contaminated waters and resistance to different treatment processes. The EPA GS chose 2 different viruses to address microbial variability and at the time the protocol was developed, they were the best studied in their variable responses to water treatment processes. However, one virus property that was not directly considered in their choice was their isoelectric points and other surface physical-chemical properties. Both test viruses have weak but different negative charges in the pH ranges of most natural source waters, with rotavirus SA11 pI = 6.4 and poliovirus type 1 pI = 7.4. Such differences in electronegativity could limit representativeness and influence the response of the viruses to a treatment technology that uses electrochemically charged filtration or adsorption media to enhance physical removal. Another limitation of the protocol now that that polio is eradicated and no longer present in most counties, inactivated vaccine is now widely used instead of live oral polio vaccine and therefore, other enteroviruses have become more important human pathogens.

The specification of *Giardia* species to be used as reference protozoan pathogens was appropriate at the time of the development of the GS Protocol, because it was considered the most prevalent waterborne protozoan and one of the most resistant to disinfection. However, since 1987, several other protozoan parasites have emerged as public health threats from drinking water exposures, in particular *Cryptosporidium parvum* and *hominus* as well as possibly *Toxoplasma gondii* and *Cyclospora cayetanensis.* As discussed above, there are substantial differences between *Giardia* and *Cryptosporidium* in the efficacy of certain water treatment processes, such as chlorination and granular medium filtration. Such differences in water treatment technology performance by type, species and strain of protozoan parasite are also likely to apply to other protozoan parasites and their bacterial surrogates, and these differences may need to be accounted for in the development and use of specific protocols.

The EPA GS choices of test microorganisms were well thought out and justifiable at the time, but the chosen organisms may no longer be conservative and representative organisms across different treatment technologies in different waters and population settings. The use of the EPA GS Protocol today presents limitations and constraints that do not adequately account for some of the more recently recognized priority pathogens, such as noroviruses and *Cryptosporidium* spp., and their potential differences and variabilities in properties and responses to treatment technologies. There are good reasons to consider amending or updating these microbial choices to better reflect the ranges of differences now observed among pathogenic waterborne microbes and how this may influence their responses to different POU/HWT technologies in a contemporary protocol for technology performance evaluation or verification. It is also noteworthy that the 1987 GS protocol was to be updated periodically every few years, but this still has not happened.

7.5.3. NSF P248: Military Operations Microbiological Water Purifiers

NSF P248 applies to portable POU systems intended to purify natural waters of unknown microbiological quality. This protocol also requires the achievement of certain bacterial, virus and protozoan parasite reductions for a water treatment device. Protocol NSF p248 requires challenge testing with any one of the following bacteria: *Escherichia coli* American type culture collection (ATCC) 11229, *Raoultella terrigena* ATCC 33257, or *Bacillus atrophaeus* ATCC 9372, the latter in its resistant spore form. The protocol specifies these bacteria because of their extensive past use in disinfection studies and other water treatment testing protocols. *E. coli* and *R. terrigena* are also chosen because of their potentially high presence in untreated surface waters, and the health significance of *E. coli* as an indicator of fecal contamination. The *Bacillus atrophaeus* spore was chosen because it shares many characteristics with *Bacillus anthracis*, which causes anthrax and is a bioterrorism threat to drinking waters.

Viruses specified for testing under this protocol are MS2 and fr, both of which are male-specific (F+) RNA coliphages. These viruses were chosen because of their small size (both <25 nm) and differing isoelectric points (pI) of 3.9 and 8.9 for MS2 and fr, respectively. These differences in pI address the possibility that electrostatic forces may influence the performance of devices using mechanical and electrostatic filtration or adsorption. However, these two test viruses may not adequately represent the many differences among human enteric viruses in response to disinfection treatment technologies such as UV irradiation or chlorination, because they are both small icosahedral viruses with single-stranded RNA genomes. Human enteric viruses are more variable in size, morphology and surface properties, and can have single- or double-stranded genomes of either RNA or DNA. Such morphological and compositional differences may result in different responses to physical, chemical and biological POU/HWT processes.

Protozoan (oo)cyst reductions are to be demonstrated with *Cryptosporidium parvum* oocysts under this protocol. *Cryptosporidium* oocysts were chosen over *Giardia* cysts because the oocysts of *Cryptosporidium* are smaller in size, which impacts removal by filtration, and they are more resistant than *Giardia* cysts to disinfection, such as by chlorine and ozone.

Although NSF P248 applies to all POU systems, the protocol seems to be targeted towards filtration devices and provides more conservative organisms overall for this type of physical treatment technology than for physical or chemical disinfection processes. This focus on filtration technologies is likely due to the applications and use settings of devices likely to seek certification under this protocol, where portability and reuse are high priorities. These use attributes and conditions apply especially to military use in the field in remote, low-resource settings. Overall, as with other POU/HWT protocols now available, additional consideration for microbial diversity and variability for various disinfection and other treatments technologies may strengthen the protocol. However, compared to the original US EPA Guide Standard Protocol, it has moved away from the use of human and simian enteric viruses, which are technically difficult and costly to produce and assay and pose biohazards when used in POU/HWT technology testing protocols.

7.5.4. NSF/ANSI Standard55: Ultraviolet Microbiological Water Treatment Systems

Until 2019, NSF Standard 55 addressed POU or POE ultraviolet systems solely using an indirect measurement of dose delivered. It requires only the indicator virus MS2 coliphage as a reference microbe and assumes predictive relationships between this well-studied virus and other viruses, as well as bacteria and protozoa, based on existing data for UV dose-response for *E. coli*, *R. terrigena*, poliovirus, rotavirus, *Cryptosporidium* and *Giardia*. Survival data for poliovirus and rotavirus [76] show that between a 3- and 4-$\log_{10}$ reduction in both poliovirus and rotavirus may be achieved by a UV dose of 30,000 uW-s/cm^2 (30 Mj/cm), while a greater than 6-log reduction of *E. coli* may be projected. Additional data [115] show a 5-log reduction of poliovirus at 40,000 uw-s/cm^2. In NSF ANSI 55 2000, a minimum UV dosage of 38,000 uW-s/cm^2 at the failsafe setpoint was set as an equivalent 4-log virus reduction requirement. To be consistent with International Standards, the minimum UV dose in NSF/ANSI 55 2002 was changed to 40 mJ/cm^2 (40,000 uw-s/cm^2) at the alarm set point. Survival data for *Cryptosporidium* [116] and *Giardia* [117] show that a minimum 3- to 4-$\log_{10}$ reduction in both *Cryptosporidium* and *Giardia* may be accomplished by a UV dose of 10 mJ/cm^2.

In 2019, there was a scope expansion to also include UV technologies that did not operate at 245 nm, by adding a direct log-reduction test with an indicator organism, coliphage Q-beta, to serve as a surrogate for all viruses. Q-beta, a male-specific (F+) RNA coliphage similar to MS2, was determined to be an acceptable surrogate for Rotavirus and the test method held true at both ends of the UV wavelength range examined (254–285 nm). This direct testing with Q-beta is simpler, more consistent and less expensive than having to conduct the collimated UV beam study of the original approach.

The original protocol will remain in NSF/ANSI Standard 55 for at least five years. Manufacturers of 245 nm technologies will have the option to evaluate their product using the new or old test method defined in the standard. For devices other than 245 nm, the new method will be required with the following criteria

- 4-$\log_{10}$ reduction of Q-beta at the alarm set point for Class A devices,
- 1.5-$\log_{10}$ reduction of Q-beta for Class B with UV source irradiance at 70% normal output, or
- 2.14-$\log_{10}$ reduction with UV source irradiance at 100% normal output.

The NSF Joint Committee meeting notes and validation data for the standard would provide any consideration of other evidence of diversity and variability in response to UV radiation treatment among other microbe types, species or strains [70,118,119]. For example, the intentional decision was to not use adenoviruses, the most resistant to UV radiation among enteric viruses, as the driver for the requirements for performance [119]. It is recommended that a further review should be done to assist in understanding the completeness and adequacy of consideration of microbial diversity and variability addressed by this protocol.

7.5.5. Brazilian NORM ABNT NBR 15176:2004

This protocol classifies and specifies the performance efficacy of a POU/HWT technology based on particle retention efficiency, efficiency in reducing free chlorine concentration, specifying a 2-$\log_{10}$ reduction minimum that does not appear to be health risk-based. There is no clear rationale for this performance target identified in the protocol.

This protocol does not adequately address microbial diversity and variability, as the only biological performance requirement is a 2-$\log_{10}$ reduction of *E. coli* bacteria, whether it is through physical removal or microbial inactivation technologies. This protocol and its microbial performance specification do not adequately account for the differences in performance efficacy likely to occur with viruses subjected to treatment by filtration and chemical disinfection, nor for protozoan parasites subjected to treatment by chemical disinfection.

7.5.6. China Ministry of Health GB/T 5750-2006 Standard and Ministry of Industry and Information Technology (MIIT) and Ministry of Health GB/T 5750-2006 Standard

The China MOH standard has two bacteriological challenge performance requirements: a 1-$\log_{10}$ reduction of *E. coli* resulting in 0 cfu/mL in test water effluent, and a reduction of total bacteria plate counts (TPC) to <100 TPC/mL in the test water effluent. There is no evidence to suggest that the target microbe and microbe reduction choices are health risk-based, and no clear rationale for the choice of test microbes or reductions was provided. However, there are primary standards for both *E. coli* and TPCs in the GB 5749-2006 Standards for Drinking Water Quality of the People's Republic of China.

The standard and protocol only consider bacteria as potential health-related waterborne microbes of concern and do not directly address waterborne viruses and protozoan (oo)cysts. Because it does not address microbial categories other than bacteria, waterborne viruses (which are of smaller size than bacteria) could give different and probably lower performance efficacy by such filtration processes. The protocol may be adequate in addressing the removal of protozoa by filtration technologies because of their larger size than the *E. coli* bacteria specified in the protocol. However, the protocol does not address the likely difference in responses between *E. coli* bacteria and protozoan parasites to chemical disinfection processes.

The China MIIT Standard for household and similar water treatment units specifies total coliform bacteria as the test microbe and a geometric mean concentration of 200–2000/100 mL in the test water. Complete removal would achieve a 2.3- to 3.3-$\log_{10}$ reduction. No other test microbes are specified in this standard. Hence, there are inconsistencies in the bacterial reduction requirements of these different Chinese technology performance evaluation documents and standards

7.5.7. Mexico Standard NOM-244-SSA1-2008

This standard and protocol of Mexico requires household treatment technologies to reduce mesophilic aerobic bacteria by 95%, and total coliforms by 99.99% (4-$\log_{10}$ reduction). There is no justification provided for the choice of test organisms or target microbe reductions, and the standard does not appear to be health-risk-based. Like the Brazilian standard and protocol, this Mexican protocol only considers bacteria as potential health-related waterborne microbes of concern, and it focuses on water filtration devices. Therefore, like the Brazilian protocol and standard, this protocol and standard does not adequately address and evaluate filtration reduction performance for waterborne enteric viruses. This protocol and standard may be adequate in addressing waterborne protozoan removal by filtration processes because of their larger size relative to bacteria like *E. coli*. However, like the Brazilian standard and protocol, the Mexican protocol and standard does not directly address the likely difference in responses between *E. coli* bacteria and either viruses or protozoan parasites to chemical disinfection processes.

In summary, it is clear that the available protocols and recommendations for the evaluation of POU technologies represent a broad spectrum of testing techniques, evaluation methods and performance standards. Additionally, none of the protocols summarized

above sufficiently address important issues such as microbial aggregation, the variation in testing between different categories of microbes, their species and their strains, as well as the viable but non-culturable (VBNC) phenomenon.

The guidelines provided in the WHO: Evaluating Household Water Treatment Options, the EPA Guide Standard Protocol, NSF P231 and NSF P248 each provide suggested test microbes and targets for bacteria, viruses and protozoan parasites. In contrast, the NSF 55, Brazilian NORM ABNT NBR 15176:2004, China Ministry of Health GB/T 5750-2006 Standard and the Mexico Standard NOM-244-SSA1-2008 do not provide targets for microbial reductions in all categories, which is a limitation to the applicability of these protocols. The NSF 55 standard is designed only for ultraviolet systems and focuses exclusively on coliphage virus testing, which is inconsistent with the currently accepted health-based approach of direct measurement of all three microbial groups. Additionally, the Brazilian protocol only addresses one category of microbial contamination in POU technologies with a 2-$\log_{10}$ reduction requirement for *E. coli*. However, there is no identified rationale for the selection of this performance target and its value. The Chinese T 5750-2006 Standard recommends only a 1-$\log_{10}$ reduction in *E. coli* as well as tests for total plate count bacteria. This protocol also does not provide information on the selection of the *E. coli* reduction target and does not include virus or protozoan performance indicators. Total plate count performance targets are inadequate for the evaluation of drinking water risks associated with microbial contamination and other methods of evaluation should be considered. The Mexico Standard NOM-244-SSA1-2008 also defines an *E. coli* $\log_{10}$ reduction but does not specify the approach taken to define this performance target. Additionally, while mesophilic aerobic bacteria counts are included as a test microbe, these bacteria do not reflect the currently accepted health-risk-based microbial performance targets and are therefore inadequate for the evaluation of POU technologies.

In response to some of the issues associated with having multiple HWT performance evaluation protocols and the different and inconsistent requirements associated with those protocols, the WHO created a joint "Harmonized Testing Protocol: Technology Non-Specific" [120]. The goal of this document is to provide study designs for the evaluation of POU technologies for the risk-based targets identified in the WHO Recommendations. The Harmonized Protocol is based on recommendations provided in the WHO Evaluating Household Water Treatment Options (2011) document, which advocates for a WHO Scheme of designated testing laboratories that will evaluate according to a Harmonized protocol the POU technologies submitted for review. This process will provide a WHO Scheme performance outcome of removal or inactivation efficiency based on the targets specified by the 2011 and 2014 documents. This new protocol focuses on the standardization of testing methods and aims to support evaluation against the WHO Scheme criteria with appropriate bacteria, virus and protozoan parasites to evaluate performance based on the accepted risk-based framework used to evaluate drinking water quality.

8. Microbial Factors Relevant to POU/HWT Performance: Microbial Aggregation

Microbial aggregation may have profound effects on the response of microbes to POU/HWT technology performance evaluation. This is because microbial aggregation results in the formation of larger particles that now contain many and varied numbers of microbes, with different particle numbers, sizes, sedimentation rates and other physical properties. Microbes within the aggregate may be physically removed differently than dispersed microbes and may also be protected from the antimicrobial effects of a disinfectant. Therefore, microbial aggregation may influence the microbial reduction responses of physical removal processes and both chemical and physical disinfection processes used by POU/HWT technologies.

8.1. Aggregation of Viruses

Enteric viruses may occur in aggregated form during the process of assembly of progeny virus particles within mammalian cells. These aggregates may consist of dozens

to hundreds of individual virions. Tightly packed intracellular crystalline arrays of enteroviruses, including polioviruses [121] and enterovirus 71 [122], have been observed by electron microscopy within continuous mammalian cell cultures. Membrane-associated aggregates containing dozens of rotaviruses were observed by electron microscopy in human and porcine stools as well as in rotavirus-infected cell culture lysates [123,124]. When infected cells collapse and release virus particles, these arrays of virions may remain at least partly intact. Consequently, it is probable that water contaminated with fecal material may contain suspended enteric viruses in various states of aggregation, possibly associated with remnants of virus-infected cells.

The phenomenon of virus aggregation is of significance to the testing of POU/HWT technologies because virus aggregates have been shown to be more resistant than single virus particles to inactivation by oxidant disinfectants such as bromine [125]. It is believed that sheltering of virions within aggregates of particles reduces or eliminates exposure to inactivating chemicals or UV radiation. It may be expected that the use of aggregated virus stocks in POU/HWT technology testing may provide greater performance challenges to treatment systems which rely on chemical inactivation by oxidants or heavy metals to inactivate viruses. Conversely, POU/HWT systems which utilize filtration may demonstrate greater virus removal efficiency when challenged with aggregated viruses. Given the belief that polluted waters serving as the sources for household POU/HWT systems likely contain enteric viruses in various degrees of aggregation, it would be more appropriate that crude virus stocks which have not been processed in such a way as to minimize aggregation should be used in POU challenge experiments. The extent to which such aggregated viruses can be produced in a consistent manner for POU testing poses challenges that need to be addressed. Mattle et al. describe methods to induce aggregation of viruses by overcoming electrostatic forces between negatively charged virus surfaces, namely by lowering pH or altering ionic strength of the test water [126]. Producing virus stocks with more defined states of virus aggregation may be possible using defined pre-filtration or centrifugation procedures and conditions to retain smaller aggregates but remove the very large virus aggregates that would not likely be present because they would settle out of the water anyway. However, a specific protocol or procedure to produce such controlled virus aggregates has not been developed and approved for use in performance evaluation of HWT technologies.

There is a role for the use of highly purified and dispersed or disaggregated virus stocks in certain types of virus inactivation studies. If experiments are being performed to determine the kinetics of virus inactivation with specific doses of disinfectants or UV radiation, particularly if the data is to be compared with results of experiments using other microbes or disinfecting procedures, a highly purified, dispersed and disaggregated virus stock would be appropriate. Extraction of virus stocks from cell cultures or fecal samples with halogenated solvents such as chloroform or proprietary hydrofluorocarbon (HFC) solvents with zero ozone-depletion potential, possibly followed by density gradient centrifugation or filtration through membranes with pore sizes which allow only single virions to pass, will yield virus stocks composed mostly of largely dispersed and disaggregated virus particles [127].

8.2. Bacterial Aggregation

Aggregation may also influence the response of bacteria to POU/HWT. Bacteria in environmental waters may be aggregated for various reasons, including the propensity for some bacteria to produce extracellular polysaccharide polymers that promote aggregation and the formation of biofilms. Numerous strains of enteric pathogenic and indicator bacteria, including species or strains of *Salmonella, Shigella, Vibrio, E. coli, Klebsiella, Enterobacter* and *Enterococcus*, can synthesize extracellular capsules consisting of tightly bound lipopolysaccharide molecules or more loosely bound and soluble microcapsular polysaccharides [128–130]. These capsule structures play important roles in pathogen–host interactions, protection against toxic hydrophobic macromolecules like bile salts present in

the digestive tract, defense against phages and formation of biofilms on surfaces [131]. The presence of extracellular capsular material can also enhance formation of loose free-floating aggregates of bacterial cells [132]. Aggregate size can be increased by both bacterial cell division and adhesive collisions with other cells or aggregates, while aggregates can decrease in size due to hydrostatic shearing and erosion. Aggregate formation might occur most readily in a laboratory batch culture containing only rapidly dividing cells of a single strain of bacteria suspended in a nutrient broth. In such a simple system, the size of bacterial aggregates is inversely related to the mixing rate, because higher hydrostatic forces prevent formation of larger aggregates [132]. Once aggregates of enteric bacteria are fecally excreted into a body of water, the size of bacterial aggregates would be expected to decrease because cell division would decline due to nutrient limitation, adhesive collisions with other cells would be unlikely and hydrostatic forces would erode and fragment the aggregates of cells. However, enteric bacteria could accumulate in aggregates with other bacteria, such as during biological water or wastewater treatment and in biofilms that form.

The role of bacterial aggregation and its effects on disinfection efficacy has been investigated. In studies of aggregating and non-aggregating isogenic strains of *Acinetobacter*, aggregation increased resistance to various potable water disinfectants by 2- to 30-fold [133]. For *Klebsiella pneumoniae* grown under low nutrient conditions to produce cell aggregates of different sizes, large aggregates were much more resistant to chloramine disinfection than were small aggregates [134].

Disinfection of aggregated bacteria has been investigated in kinetic studies of bacteria reduction by chlorine at doses used in water treatment [135]. An *E. coli* strain with O-agglutination activity and *Salmonella anatum*, both of which produced aggregates, exhibited longer survival periods than similar non-aggregating bacteria. Furthermore, disinfection kinetics differed between *E. coli* and *S. anatum*. Response to chlorination by similar bacteria possessing the H-agglutinin resulted in disinfection kinetics that were intermediate between the values for the non-aggregating bacteria and those that aggregated. The rate of bacterial disinfection was found to be a function of oxidation-reduction potential. In studies on coliform bacteria associated with different size particles recovered from sewage, coliforms associated with the <7 μm fraction were inactivated more rapidly than coliforms associated with the >7 μm fraction by 0.5 mg/L chlorine dose of buffered water at 5 °C and pH 7 [136]. Homogenization of the >7 μm fraction not only resulted in an increase in the number of <7 μm particles, but also increased the rate of inactivation to a rate similar to that of the less than 7 μm fraction. Results of these various studies suggest that chlorine concentrations used in water treatment practice are less able to kill bacteria within aggregates, and that colony counts do not reliably quantify bacterial counts if bacteria are present as aggregates. Aggregation is thought to be a common occurrence in natural waters, but the degree to which aggregates are present at any given time is difficult to quantify and may readily change.

Biofilm formation occurs widely on surfaces, such as the pipes of drinking water distribution systems and the media of biologically active porous filters, such as granular activated carbon and slow sand filters. Such biofilms can release aggregates of cells into water to become a source of aggregated bacteria. In studies that compared chlorine disinfection of *Burkholderia cepacia* and *Pseudomonas aeruginosa* grown as discrete cells or as aggregates of different sizes, disinfection resistance generally increased with a higher percentage of larger cell clusters and detached biofilm [137]. Samples with a lower percentage of large clusters were more easily disinfected.

It has been suggested that the presence of extracellular capsule macromolecules surrounding bacterial cells would provide protection against disinfecting chemicals even for individual non-aggregated cells. However, when the sensitivity to free chlorine and monochloramine of an encapsulated strain of *Klebsiella pneumoniae* was compared with that of a non-encapsulated mutant that produced 55 times less extracellular macromolecules than the mutant, there was no significant difference in the resistance of the two strains to either free chlorine or monochloramine [138]. Hence, the results of this study indicate

that encapsulation of individual cells may not always provide added protection against chlorine disinfection. However, in free chlorine disinfection studies of "smooth" *Vibrio cholerae* strains that do not produce an extracellular polysaccharide layer compared to the "rugose" strains that do, the disperse rugose strain cells were similar in inactivation kinetics to the smooth strain cells, but the aggregated rugose strain cells were much more chlorine-resistant [67].

The effect of aggregation on bacteria response to UV disinfection has also been studied. The impact of natural aggregation was assessed for total coliform bacteria in natural surface water samples [139]. Dispersion of total coliform natural aggregates by blending prior to UVA exposure resulted in enhanced inactivation rates as compared to untreated water. However, removal of particles by 8-micron membrane filtration did not improve UVA disinfection efficiency. In studies of the effect of aggregation of spore-forming microbes such as *Bacillus subtilis*, aggregation of both laboratory strains and environmental samples increased resistance to UV disinfection [70].

Some waterborne bacteria such as *Mycobacterium* species that naturally aggregate may be protected against disinfection processes. In secondary effluent spiked with *Mycobacterium terrae* [140] and sequentially filtered through 100, 41 and 20 μm filters to select for different aggregate sizes that were then exposed to doses of UV light (10 to 60 mJ/cm^2 at 254 nm) and free chlorine (concentration x contact time values of 27 to 150 mg-min/L at 4 °C), *M. terrae* inactivation in wastewater was initially rapid, with 2.5-log reduction at 14 mJ/cm^2 UV dose and 56 mg-min/L free chlorine contact time (CT) [140]. However, in unfiltered and 100-micron filtered samples, spiked *M. terrae* were still present in the highest UV and free chlorine doses evaluated. Interestingly, *M. terrae* passing through 41- and 20-micron filters were inactivated rapidly, with no survivors after moderate disinfection doses. The results of this study suggest that UV inactivation of mycobacteria in wastewater may be compromised by aggregates larger than 41 μm. Therefore, aggregate size can also influence disinfection efficacy.

Studies examining enteric bacterial elimination from water by various treatment processes typically have not utilized specific procedures to discourage or eliminate aggregates. Bacteria grown in broth cultures are often sedimented out of suspension by centrifugation and resuspended, sometimes after several repeated washings. These processes might be expected to encourage formation of bacterial aggregates because the resulting bacterial pellets are merely resuspended in a buffer or test water by shaking or vortexing for a short time until visible clumps are broken up. Two studies specifically compared the response of *Klebsiella pneumoniae* and *E. coli* to chloramines [134] and chlorine dioxide [141] respectively, before and after centrifugation, rinsing and resuspension in buffers to reduce oxidant demand, and found no difference in response to disinfection. This suggests that pelleting the cells did not result in enough increased aggregation to provide a protective effect against these oxidant disinfectants. These observations provide useful information on how bacteria cells can be prepared consistently for POU/HWT performance evaluation studies.

8.3. Aggregation of Protozoan Parasites

Aggregation of protozoan parasites and its effects on their response to disinfection and other water treatment processes has been studied to some extent. It has been reported that protozoans capable of aggregating were more resistant to disinfectant agents than protozoans incapable of aggregating [142]. However, the extent to which cysts or oocysts of enteric protozoan pathogens are present as aggregates in water to be treated for drinking is largely unknown. This is because the processes for recovering and concentrating these organisms from water involve multiple physical and chemical treatment steps that can change the state of aggregation (such as centrifugation, resuspension in buffer with surface active agents and immunocapture on and then elution from a solid substrate). The formation and excretion of oocysts as individual particles does not necessarily lend itself to oocyst aggregation. A study examining the surface chemistry of *Cryptosporidium parvum* oocysts found that little oocyst aggregation occurred in water at neutral pH and low salt

concentration [143]. Similarly, Iyer et al. found that in waters with low salt concentrations, there was little aggregation of *C. parvum* oocysts, but the addition of bivalent or trivalent cations increased aggregation due to the suppression of surface charges [144]. Studies documenting protozoan cyst physical removal and disinfection typically use purified (oo)cysts that are produced from feces of infected animals by utilizing a series of purification and concentration procedures, such as centrifugation and chemical extraction and flotation in dense media or density gradients, in order to produce stocks in which cysts are known to be disaggregated. Because such preparations tend to contain dispersed cysts and oocysts, they may be well-suited for POU/HWT performance evaluation studies.

8.4. Microbial Aggregation as Addressed by Testing Protocols

The degree to which each POU/HWT technology performance evaluation protocol or standard considers and addresses the issue of microbial aggregation is provided here. The EPA 1987 protocol recommended that large clumps of bacteria be removed from seed stocks by filtration through Whatman #2 filter paper. This is a qualitative grade cellulose paper with a nominal pore size of about 8 µm. Because the recommended test bacterium, *Raoultella terrigena*, is about 2.0 µm long and 0.5 µm wide, it is expected that only small clumps of a few cells would pass through this filter. The EPA protocol also recommends that virus seed stocks in cell lysates be purified by extraction with an organic solvent like chloroform and sucrose density gradient centrifugation. Selection of density gradient fractions that contained predominantly single particles would give primarily monodispersed viruses in the seed stock.

The NSF Protocol P231 and the NSF/ANSI Standard 55 for UV systems both follow the recommendations of the 1987 EPA protocol. While the P231 protocol makes no changes or additions, the Standard 55 explicitly states that each challenge microbe for UV collimated beam disinfection testing be a "monodispersed suspension". However, the detailed propagation and preparation methods provided for the various challenge microbes used to validate this standard (the yeast *Saccharomyces cerevisiae* in place of a bacterial species, plus phages MS2 and T1) did not include any procedural steps that would yield monodispersed microbe stocks.

The Chinese protocol, Guideline Relating to Drinking Water, included only total coliform bacteria and fecal coliforms as possible challenge microbes. Because no bacterial challenge stock preparation procedures were provided, aggregation was not addressed.

The NSF Military Protocol P248 explicitly states that it follows the procedures recommended by the 1987 EPA protocol and the NSF Protocol P231. However, when providing relatively specific instructions for preparing the bacteria challenge stock, it omits the filtration step of the phosphate buffer-washed bacteria that would remove large aggregates of bacterial cells. Protocol P248 calls for the production of the challenge stock of coliphage MS2 or coliphage fr by the sloppy agar method, followed by centrifugation to remove bacterial debris and serial filtration through Whatman #2 filter paper, a 0.45 µm membrane filter and a 0.1 µm membrane filter. This would remove large aggregates, as MS2 and fr are about 27 and 23 nm in diameter, respectively.

The Chinese Ministry of Health procedure and the Brazilian protocol did not address the issue of bacterial aggregation at all. In the Mexican procedure, *E. coli* cells grown on agar are washed off the agar layer with saline and gently shaken before being added to the test water, leaving open the possibility that the *E. coli* would be present in the influent test water in clumps (aggregates) of cells.

Overall, the role of aggregation in the response of microorganisms to POU/HWT treatment and water treatment in general for performance evaluation has not been systematically studied, and the effects of aggregation are poorly characterized and uncertain. Therefore, the role of aggregation should be more carefully addressed in performance evaluation of both physical removal and microbial disinfection (inactivation) POU/HWT treatment technologies. It may be that use of both dispersed and aggregated microbes should be recommended in performance evaluation of POU/HWT treatments. However,

it is first necessary to ascertain the effects of the dispersed and aggregated states of test microbes on their reductions by both physical removal and microbial disinfection processes of POU/HWT treatment.

9. Microbe–Particle Association

In drinking water treatment, microbes can be physically protected from disinfection processes by attachment to or association with suspended organic and inorganic particulates in the water. In addition, the association of microbes with particles in water influences their removal by filtration, sedimentation and other physical processes. Small microbes such as viruses that have become associated with larger particles may be removed to a much greater extent than discrete virus particles by physical treatment processes such as filtration and sedimentation. The removal of viruses from water by chemical coagulation-flocculation, sedimentation and then filtration is largely due to the association of the viruses with the chemical floc particles that are produced in these processes [145–148].

The extent to which microbes of public health concern in ambient waters are associated with other particles has been studied. For example, several bacterial, protozoan and viral indicator organisms in storm waters and surface waters have been investigated for their settleability (sedimentation) due to particle association in both storm and dry weather conditions [149]. The fraction of organisms associated with settleable particles in stormwater varied by type of microbe, and their partitioning behavior generally changed between dry weather and storm conditions. For the bacterial indicators of fecal coliforms, *E. coli* and *enterococci*, an average of 20–35% were associated with settleable particles in background samples and 30–55% in storm samples. *Clostridium perfringens* spores exhibited the highest average particle association, with storm values of 50% to 70%. Coliphage partitioning was more variable, with 20–60% associated with settled particles during storms. In a similar study, the partitioning behavior of two protozoan pathogens, *Cryptosporidium* and *Giardia*, and several fecal indicator organisms (fecal coliforms, *E. coli*, *enterococci*, *Clostridium perfringens* spores and coliphages) were characterized and compared in natural waters under both dry and wet weather conditions. Partitioning behavior indicative of particle association varied by microbial type, with 15–30% of bacterial indicators (fecal coliforms, *E. coli* and *enterococci*) associated with settleable particles compared to 50% for *C. perfringens* spores. Both protozoan pathogens exhibited similar levels of particle association during dry weather (roughly 30%), with increased levels observed during wet weather events (*Giardia* to 60% and *Cryptosporidium* to 40%). The settling velocities of particle-associated microbes were also estimated, and those of bacterial indicators (fecal coliform, *E. coli* and *enterococci*) and *C. perfringens* spores were found similar to that of *Giardia* and *Cryptosporidium* [150]. There are many complex processes and properties of microbes that influence microbe attachment to suspended particles in water, several of which are described here. These phenomena have important implications for POU/HWT performance evaluations and the choice and preparation of testing materials.

9.1. Particle Behavior

Enteric viruses typically possess diameters of 25 to 100 nm. Bacterial cells of the common rod-shaped enteric species *E. coli* are about 2 μm long and 0.5 μm in diameter. Oocysts of the protozoan pathogen *Cryptosporidium parvum* are 4 to 6 μm in diameter, cysts of *Giardia* spp. are about 7–12 μm in diameter and cysts of *Entamoeba histolytica* are about 10–20 μm in diameter. All of these microorganisms are considered to behave as colloidal particles in water, because their settling velocity due to gravity is so slow that particles of this size remain suspended in the water column [151]. Inorganic particles smaller than 10 micrometers, for example, clays, metal carbonates, hydroxides and oxides, and amorphous organic detritus particles, also exhibit colloidal behavior in the water column. Larger particles may eventually settle out into the sediment but may become resuspended in the water column during rainstorm events and other changes in weather (such as turnover in

lakes and reservoirs due to temperature destratification) or as the result of human activities (e.g., agriculture, aquaculture, fishing, dredging and impoundment management).

Microbes possess molecules on their outer surfaces which may be ionized in contact with water, giving microbial particles surface electrochemical charges. For example, protein molecules possess both amino groups (NH_2) which can accept a proton and become positively charged (NH_3^+), while carboxyl groups (COOH) can donate a proton, becoming negatively charged (COO^-). The extent to which the various groups become ionized is controlled primarily by the pH and the ionic composition of the body of water. As a result of this process, microbes and inorganic particles usually possess a net charge, usually negative [109,151,152], in natural waters.

The outer layer of water molecules surrounding a particle contains a surplus of ionized atoms and molecules of the opposite charge sufficient to neutralize the charge of the particle. The thermal motion of water molecules and large-scale mixing of the water column causes particles to collide with one another, with the concentration of the particles and the mixing rate of the water influencing the frequency of particle-to-particle collisions. If particles possess net opposing charges, they may attract and attach to one another. If, however, both colliding particles possess net positive or net negative electrostatic charges, the two particles will repel one another.

In addition to electrostatic interactions between particles, there is an attractive force, called the London-van der Waals force, which operates within a much narrower range than the electric double layer [151]. This force is a result of the formation of attractive oscillating dipolar regions within the electron cloud of electrically neutral atoms and molecules. If, for example, the electric double layer between two particles of the same net negative charge is compressed by the presence of multivalent ions such as magnesium (Mg^{2+}) or calcium (Ca^{2+}), to such an extent as to allow the particles to approach one another closely enough for the attractive London-van der Waals force to predominate over opposing electrostatic forces, the particles can attach to one another. Hydrophobic interactions also influence microbe interactions with particles, including other microbes, and surfaces and their response to treatment processes [153–155].

As a consequence of these complex interactions, microbes may become associated with suspended particles in natural waters to varying degrees. Seeding of untreated sewage influent with polioviruses and subsequent recovery demonstrated that 28% of polioviruses associated with particles greater than 0.3 µm in diameter [156]. Examination of particle size fractions of recently settled sediments for coliphage viruses from constructed wetlands and detention ponds designed to collect urban stormwater found that the largest proportion of coliphages (24% to 50%) were detected in the finest particle fraction, less than 2 µm, the same fraction that would most readily be resuspended in the water column during storm activity [157]. An investigation of coliphage virus partitioning in stormwater found that 20–60% of coliphages became associated with particles large enough to settle into the sediment [149].

Analysis of stormwater for the indicator bacteria *E. coli*, fecal coliforms and enterococci found that about 65% to 85% of the bacteria remained suspended in the water column, while the remainder became attached to particles large enough to settle out [158]. Most of the bacterial cells which did become particle-associated were attached to the smaller particles of 5 to 30 µm in size. Another study examining microbial association with particles in stormwater found that 30–55% of most types of indicator bacteria were associated with particles large enough to settle out of the water column [149].

Investigations of the association of *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts with particles have found that oocysts and cysts are negatively charged in freshwater conditions and can aggregate with other oocysts or cysts or become associated with other particles and settle out of water to varying degrees, depending on water quality and hydrological conditions [150,159,160]. The tendency of oocysts and cysts to become associated with settleable particles tends to increase during storm events with much higher suspended particle loads.

9.2. Responses of Particle-Associated Microbes to Water Treatment Processes

The association of enteric microbes with colloidal inorganic and organic particles has been shown to provide a protective effect when microbes are exposed to antimicrobial processes or conditions. Total coliform and fecal coliform bacteria survived exposure to ultraviolet light when associated with organic particles ranging in size from 8 to 70 μm in sewage, better than coliforms in sewage filtered to remove the organic particles [161]. *E. coli* cells released into mesocosms in a lake exhibited longer survival when associated with particulate matter, primarily algal cells [162]. The particle-associated cells were partially protected from predation by grazing rotifers and flagellates. There was also evidence that nutrient levels which could be utilized by *E. coli* were higher at particle surfaces. Nontronite, an iron-rich clay, present in groundwater at a concentration of 500 ppm, reduced poliovirus inactivation when exposed to solar radiation [163]. Polioviruses present within fecal solids smaller than 5 μm were protected from inactivation by chlorine, an effect ascribed to the inability of chlorine to penetrate the fecal particles and come into contact with the poliovirus particles [164]. Coliphage virus MS2 was more resistant to disinfection by chlorine when the virus particles were adsorbed to bentonite clay particles [165]. Hepatitis A virus (HAV) associated with host cells was more resistant than dispersed HAV to inactivation by free chlorine and chloramines in water [166].

9.3. Consideration of Particle-Associated Microbes by POU Testing Protocols

Because enteric microbes may be associated with fecal solids when introduced into surface waters, or may become adsorbed to inorganic or organic colloidal particles present in the water column, and because microbe adsorption to particles may provide a protective effect, the inclusion of such particles in the water for POU/HWT technology testing would be highly appropriate to realistically model prevailing conditions for surface waters likely to be the sources of water treated in POU/HWT systems. Particles can be added in the form of purified clays, commercial "test dust" products or partially treated sewage effluents. Particle-associated microbes may be expected to be eliminated more readily from POU/HWT systems which utilize filtration or other physical removal processes for treatment but might pose more of a challenge to POU/HWT systems which rely primarily on chemical or UV disinfection processes.

Because of its influence on the effects of water treatment processes in removing or inactivating microbes from water, the use of particles in test waters and the extent to which microbe–particle associations are recognized and addressed by each of the relevant POU/HWT performance evaluation protocols and standards is considered here. The 1987 EPA GS protocol recommends that challenge water matrices for POU/HWT systems using halogens, UV radiation or filtration should possess turbidity levels of at least 30 nephelometric turbidity units (NTU). This level of turbidity is approximately the same as secondary sewage effluent and as surface waters following heavy rain. Tap water can be supplemented with particulate matter in the form of a commercially available "test dust", originally developed to test the efficiency of equipment air filters. The fine-grade formulation of crystalline silica "test dust" contains inorganic particles ranging in size from about 1 to 175 μm and is composed of naturally occurring quartz and cristobalite.

The NSF Protocol P231, which follows the testing procedures recommended by the EPA protocol, makes no changes or additions regarding the use of turbidity particles or their sources. The NSF/ANSI Standard 55 for testing UV systems, which also follows the EPA protocol, specifies, however, only a general test water with a turbidity of less than 1.0 Nephelometric Turbidity Units (NTU) that is to be used for the collimated beam test. No other higher turbidity challenge waters are recommended.

The Chinese protocol, Guideline Relating to Drinking Water, mentions turbidity only in terms of the upper limits of 0.5 or 1.0 NTU allowed in the POU product water.

The NSF Military Protocol P248, which is based on the EPA and NSF P231 protocols, specifies that test waters used to challenge POU systems utilizing halogens, UV light or filtration to purify water must possess turbidity in the range of 30 to 50 NTU. The protocol recommends that a commercial test dust of fine grade be used to adjust the turbidity.

The Chinese Ministry of Health protocol recommends using distilled water as the test water, and no particulate matter is added, which rules out any microbe–particle effects during testing. Likewise, the Brazilian protocol recommends aging of water and use of challenge waters of less than one nephelometric turbidity unit with no addition of particulate matter. The Mexican testing protocol recommends using water from a public water supply for testing, implying that low-turbidity water is to be used. The Mexican protocol does, however, state that water quality parameters may be adjusted to challenge the POU system being tested at the discretion of the testing personnel.

10. Physiological State of Bacteria

The physiological state of bacteria has been shown to have an appreciable influence on their survival and transport in the environment and their response to water treatment processes and other environmental stressors. Bacterial cells also differ in their response to treatment processes and other environmental stressors, depending on which stage of growth they have been in, either log phase or stationary phase. Factors contributing to the physiological differences in bacterial cells include physiological changes induced by nutrient starvation (starvation-survival phenomenon) [167], the development of the viable but non-culturable (VBNC) state by exposures to various environmental stressors, including water treatment processes such as disinfection [168], and the development of filamentation or cell elongation [169] in stressful environments as documented physical and physiological changes.

10.1. Changes in Bacterial Physiology in Response to Environmental Conditions

Bacterial cells which are growing and reproducing in a nutrient-rich and favorable environment are synthesizing a wide variety of proteins, nucleic acids and lipid membrane components. In actively growing *E. coli* cells, for example, about 1000 genes are being expressed [170]. When enteric bacteria are excreted from their warm-blooded hosts into the external environment, they face drastically different conditions. The cells are exposed to nutrient limitations and other potentially stressful, injurious and lethal environmental factors, including sunlight, unfavorable osmotic conditions, fluctuating temperatures, antagonistic enzymes or heavy metals, all of which can cause nonlethal and lethal physiological and structural injury to cells. In response to cellular damage, dramatic changes occur in gene expression, cellular structure and metabolism. Bacteria have evolved genetically controlled mechanisms to limit and repair damage. In *E. coli* and in *Enterococcus faecalis* [171], most of the genes being expressed during periods of growth are repressed, and a different group of 50 to 100 genes are expressed. The resulting protein products are active in the altered configuration of the bacterial chromosome, resulting in the repression of gene transcription as well as protecting the chromosome from additional stress-related damage. Another protein combines with and brings about the formation of ribosome dimers, which protects ribosomes from digestion by cellular enzymes. The preservation of ribosomes allows early protein synthesis if the cell subsequently encounters conditions favorable for growth. Certain proteins synthesized by stressed cells alter nutrient metabolic pathways, or are significant in the protection of the cell from external detrimental conditions [172]. Other proteins regulate the self-digestion of cellular components, a process which provides a starving cell with energy and an internal source of carbon.

Bacterial cells recently exposed to stressful conditions may continue to divide by utilizing chromosomes already synthesized prior to the onset of stress [172]. The termination of normal macromolecular synthesis following the onset of stress, however, may result in

the formation of smaller cells. Nutrient-starved cells may become further shrunken due to endogenous degradation of the structural components of the cell. These morphological changes yield cells with increased surface-to-volume ratios and may have an impact on disinfection and/or physical exclusion efficacy.

Bacteria which possess the most extreme response to stressful conditions are those which can form spores, structures which are much more resistant to damaging environmental stresses than vegetative cells. *Clostridium perfringens* is an enteric species which is utilized as an indicator of fecal contamination. The spores of *C. perfringens* are used in the investigation of water and wastewater treatment processes because their resistance to some disinfection processes and to physical removal by filtration processes is similar to the oocysts of *Cryptosporidium*, an enteric protozoan parasite [63,173].

While bacterial spores do provide greater protection than vegetative cells from deleterious environmental conditions, spores can also be injured by the same causative agents and processes [174]. Consequently, the phenomenon of bacterial injury and repair and its possible significance in the testing of point of use water treatment systems should be extended to bacterial spores and their potential use as challenge organisms.

Stocks of enteric bacteria used in laboratory investigations of the kinetics and extent of water and wastewater treatment and disinfection, including POU/HWT drinking water technology testing, are almost always produced by batch culture methods due to the simplicity of the procedure. A moderate number of bacterial cells are inoculated into a vessel of an appropriate culture broth and incubated at a constant temperature for several hours or overnight, often with physical mixing. Periodic enumeration of the bacterial population in a broth culture reveals a characteristic pattern of growth, consisting of a short initial lag period with no significant increase in bacteria numbers, followed by a "log phase" growth period during which the number of cells increases exponentially, and then a "stationary phase" in which the number of cells remains relatively constant. The assumption is that the stationary phase is a consequence of the exhaustion of nutrients by the greatly increased population of bacterial cells and the accumulation of inhibitory metabolic waste products. Cells used in laboratory disinfection experiments are sometimes collected from a broth culture during the log phase, as the majority of these cells are presumed to be actively growing and healthy. Cells for disinfection experiments are sometimes not collected in stationary phase broth cultures because they are considered to be stressed due to nutrient starvation and undergo many metabolic and morphological alterations characteristic of stressed cells. Therefore, in some protocols, they are considered compromised and inappropriate for disinfection kinetics experiments. However, some investigators promote the use of stationary phase cells, as they may be more like the cells in human and animal feces that have been released into the environment and are no longer in a hospitable environment for continued growth. In this respect, such cells may be more representative of those found in fecally contaminated water, which are often no longer in a log phase growth state.

It should be kept in mind that the pattern of bacterial population dynamics exhibited by a batch culture is highly artificial in a closed system which is probably not representative of either the gut of a warm-blooded animal or a body of water contaminated by released fecal material. Some investigators have utilized chemostats as a source of bacterial stocks [175,176]. A chemostat is a more open laboratory system which permits the growth of microbes at a particular growth rate in a physiological steady state by providing a constant flow of nutrients into the system while regulating the temperature, dissolved oxygen and pH of the system, and removing metabolic waste products and a fraction of the bacterial cell population. Chemostat-grown cells are not used in current POU/HWT performance evaluation standards, protocols or guidance.

10.2. Response of Nutrient-Stressed Bacteria to Water Treatment Processes

Investigators have compared the sensitivity of enteric bacteria harvested at different physiological states to disinfectants. *E. coli* K12 and *E. coli* 0157:H7 cells harvested at the

stationary phase were more resistant to chlorine disinfection than cells collected during the lag phase or exponential growth phase [177,178]. *Enterococcus faecalis* cells forced into the stressed "stationary phase" state by glucose limitation exhibited elevated resistance to chlorine compared to actively growing cells [179]. The resistance of *E. coli* to UV-C radiation varied with the rate of growth in a chemostat, with "stationary phase" or slowly growing cells having greater resistance than cells growing at a more rapid rate [176]. However, when the resistance of *Klebsiella pneumonia* to chloramines was studied, there was no significant difference in resistance between stationary phase cells and cells harvested during the log phase [134]. But when *K. pneumonia* was grown in a highly diluted culture medium, the nutrient-limited cells exhibited increased resistance to chloramines.

The studies reviewed above lead to the conclusion that bacterial cells which have experienced the transition to the injured state, the starvation-survival state or the VBNC state, due to nutrient starvation or the effects of other environmental stresses, exhibit increased resistance to a variety of other deleterious environmental conditions as well. Therefore, it would be expected that enteric bacteria excreted from their host into the external environment, such as a body of water used as a source of drinking water, would subsequently experience changes that result in a different physiological state, accumulate cellular damage and undergo other alterations in morphology, gene expression, metabolic functions and transport activities. Consequently, it would be reasonable to use stressed bacteria when testing POU/HWT technologies and systems as these would be more representative of the state of cells of fecal origin subsequently present in environmental waters. Because it is likely that the physiological response of bacteria varies with the form and intensity of stress [172], it would be advisable to utilize a standardized stress condition for POU/HWT technology testing. The batch broth culture method will likely remain the method of choice for the production of bacterial cell stocks for POU/HWT technology testing in challenge studies using seeded test water. Therefore, the use of controlled and repeatable batch broth culture conditions and the harvesting of cells which have attained the stationary phase (as measured by turbidity) for a defined time period, for example several hours, might be a reasonable and replicable choice of test bacteria. However, use of stationary phase cells for POU technology performance evaluation may need further evaluation and characterization in order to develop and define preferred methods to obtain and use such cells having defined and reproducible properties for such studies. The use of bacteria already present in the environment, such as in raw sewage, may also be considered as more representative of the physiological states of enteric bacteria in fecally contaminated waters. However, the heterogeneity and variability of such field-harvested rather than lab-grown bacteria may pose problems in achieving consistent bacterial reduction responses to POU/HWT technologies in performance evaluation testing studies. Some consideration may need to be given to harvesting and processing such existing bacteria from wastewater in order to create representative mixed cultures of cells with definable physical and physiological states as seed stocks for POU technology performance evaluation.

10.3. Consideration of the Physiological Condition of Bacteria by POU Testing Protocols

The degree to which the physiological state of bacteria is recognized and addressed by each POU/HWT performance protocol or standard testing method shows considerable variability and often lack of any consideration at all. The EPA 1987 Guide Standard and Protocol specifies that coliform bacteria being used in point-of-use system testing should be grown overnight as a broth culture or on agar slants. This would yield bacterial cells in the stationary phase of growth, with a substantial fraction of the cells exhibiting various degrees of stress due to nutrient limitation. Cells harvested from agar plates may be aggregated to unknown extents, depending on how they are recovered and handled. No changes in these specifications are recommended in the NSF Protocol P231. The NSF/ANSI Standard 55 for testing UV HWT systems, which originally utilized a yeast, *Saccharomyces*

cerevisiae, instead of bacteria, recommended batch cultures grown for 24 h at 25 °C, likewise yielding late growth phase and stationary phase yeast cells.

The Chinese protocol, Guideline Relating to Drinking Water, does not provide any specific procedures for the preparation of total coliform bacteria to be used in the test protocol.

The NSF Military Protocol P248, following the EPA protocol, recommends growing overnight cultures of coliform bacteria at 35 °C, yielding late log phase to stationary phase cells. Likewise, the Chinese Ministry of Health protocol, the Brazilian protocol and the Mexican protocol recommend the use of overnight *E. coli* batch cultures grown in broth or on agar, yielding cells in the stationary phase and possibly aggregated.

The WHO document, Evaluating Household Water Treatment Options, states that challenge cultures of bacteria should consist of unstressed cells. However, the bacteria are to be grown in broth cultures overnight, which would yield cells in the stationary growth phase, a fraction of which may be injured because of nutrient limitation and metabolic waste exposure. Furthermore, the document states that indicator bacteria and bacterial pathogens are likely to be injured once excreted into surface waters, so the use of injured cells may be more realistic. The choice of uninjured bacterial cells as the most appropriate candidate for POU testing is due to the need for consistency. Given the wide range of forms of bacterial injury and differences in physiological state, it may be difficult to replicate injured cells of a particular physiological condition. Therefore, it may be assumed that the use of cells grown overnight, if done consistently, would provide cells in late log phase or early stationary phase that have experienced some degree of injury or stress that is reproducible and consistent in its effects on the cells if carefully controlled and specified. However, there is a need to further explore approaches to consistently and reproducibly develop microbial stocks in the lab that as far as possible resemble the physical and physiological states of the microbes in water and wastewater environments.

11. Bacterial Injury and Water Disinfection Processes

The processes and rates by which microbes are inactivated differ among microorganisms, and several inactivation (disinfection) kinetic models have been developed to describe reduction of culturable microorganisms in water when exposed to a disinfecting treatment process. Inactivation kinetics are sensitive to several factors within a system, including microorganisms and their physiological and physical states, type of disinfectant and various environmental and water quality conditions. The most commonly used model to describe water disinfection processes is the Chick-Watson model. This model is based on first-order kinetics of inactivation and asserts that microbial inactivation is a log-linear function of the disinfectant concentration and contact time (Ct). The Chick-Watson model has been successful in describing inactivation kinetics between several microorganisms and disinfectants but may be insufficient to describe the heterogeneous nature of microbial populations found in the environment. In some disinfection processes, a decreasing effectiveness of the disinfectant is observed after a certain amount of disinfectant concentration or contact time. This phenomenon, known as tailing, can occur in both physical and chemical disinfection, and is thought to be caused by factors such as microbial shielding or aggregation and the presence of microbial subpopulations with differential resistance to the disinfectant [180,181]. Multi-hit disinfection kinetics models such as the Hom model and One Hit-Two Population model account for such deviations from first-order kinetics. For more information on disinfection kinetics, the reader is directed to the following References [180,182,183].

Though disinfection kinetic models have been useful and applicable in the field of water treatment, they do not adequately account for bacterial injury by disinfection processes. Bacterial cells exposed to antimicrobial or disinfection processes in drinking water POU/HWT systems may survive the treatment process but could have suffered injury or be in an altered physiological state. This bacterial injury can result from either physical or chemical disinfection and has been defined as sublethal structural or physiological effects of exposure to debilitating processes or conditions which result in the inability of the injured

cells to reproduce under culture conditions which otherwise support the reproduction of uninjured cells [184]. Debilitating chemicals include oxidizing agents like chlorine, iodine or bromine, and heavy metals like silver, copper or zinc. Exposure to ultraviolet radiation or sunlight is an example of physical POU processes which can produce injured bacterial cells. In the case of bacteria exposed to stressors such as disinfectants or sub-lethal environmental conditions, viable but (conditionally) non-culturable (VBNC) cells are produced. The VBNC cells require modified culture conditions which allow them to repair damaged cellular components and subsequently regain the ability to reproduce under the selective culture conditions normally used to detect and enumerate indicator or pathogenic bacteria. The failure of disinfection kinetic models to account for injured bacteria could result in overestimation of the effectiveness of a disinfection treatment and subsequently allow for injured and resuscitated VBNC bacteria to be present in drinking water post-treatment.

11.1. Prevalence of Bacterial Injury

Studies by many investigators have documented injury of fecal indicator bacteria in water and foods [185–187]. Studies analyzing samples of wastewater and natural waters for indicator bacteria using selective and nonselective culture methods in parallel have revealed that up to 90% of the indicator bacteria are injured and are detected only under nonselective culture assay conditions [184,188]. The evidence that injured pathogenic bacteria may retain the ability to cause disease is important because it has implications for estimating health risks posed by bacteria exposed to injurious treatments or conditions [189]. An *E. coli* strain injured by exposure to sunlight while suspended in seawater retained its ability to produce enterotoxins [190]. *Shigella dysenteriae* cells injured by nutrient starvation maintained the shiga toxin gene and biologically functional Shiga toxin and its ability to bind to intestinal cell cultures, although it lost its ability to invade those cells [191]. *Vibrio vulnificus*, which had been induced into the VBNC state by low-temperature exposures, continued to cause mortality in mice following resuscitation within the host, although the rate of virulence began to decline after 3 days at low temperature [175]. Several strains of *Salmonella typhimurium* and *Salmonella gallinarum* that were metabolically injured by freezing or freeze-drying produced mortality in mice at rates equivalent to uninjured cells [192,193].

11.2. The VBNC Hypothesis

In the course of the investigation of bacterial injury and resuscitation, Colwell, Xu and others have developed the hypothesis that the VBNC state of bacterial cells is not simply a transitory injured condition during which bacteria lose particular capabilities due to cellular damage but is a genetically programmed state which allows bacteria to survive unfavorable conditions [194,195]. This interpretation of the VBNC state asserts that it is a distinct inducible living stage which evolved as a survival strategy for bacteria which cannot form spores [196]. Evidence providing details of the mechanisms of the VBNC living stage and its genetic control is limited and controversial. Those who reject the VBNC life stage hypothesis believe that the bacterial injury state is a temporary condition that can be manifested or described in a variety of phenotypic ways. Injured bacterial cells either repair themselves if conditions subsequently permit it or reach the point where the accumulated damage no longer allows recovery and multiplication. Experiments have attempted to demonstrate that injured enteric bacteria do not possess a distinct VBNC life stage [197–199]. Hence, the validity of VBNC as a distinct physiological state remains uncertain, with proponents that insist on its validity as a definable condition and others who believe it has no unique or distinct mechanistic basis that makes it distinctive as a stress or injury response phenomenon or condition [168,200–202].

Nevertheless, the phenomenon of bacterial injury and subsequent recovery for culturability is significant for the testing of drinking water supplies and their distribution and storage systems for indicator and pathogenic microbes, especially those in which there has

been water treatment (and disinfection in particular), whether at POU or otherwise. This is because bacterial injury and the potential for subsequent recovery leading to growth poses the possibility of false-negative culture assay results that may overlook potential waterborne risks to public health. The failure to account for bacterial injury and repair may likewise result in the over-estimation of the efficiency of POU/HWT systems to eliminate bacterial indicators and pathogens. Lack of or reduced culture-based detection by some assay procedures may not adequately indicate the ability of the injured bacteria to grow and reproduce under other culture conditions or to infect a host.

11.3. Mechanisms of Bacterial Injury

Investigations of the specific nature of injury of bacterial cells have uncovered several mechanisms. Solar or UV radiation can damage the bacterial chromosome by causing formation of bonds between adjacent thymine bases on the same strand of DNA, distorting the conformation of the DNA double helix, and interfering with protein synthesis [115]. Exposure to sunlight or UV can also produce hydrogen peroxide and other reactive oxidative species which can damage DNA and proteins [203]. Chlorine compounds may likewise damage cells by toxic oxidative attack [204], with a major site of chlorine damage appearing to be at the cell membrane, where the enzyme complex that synthesizes ATP is located, resulting in the disruption of energy production [184]. However, other investigators explored the effects of chlorine on *E. coli* and *Pseudomonas aeruginosa* bacteria by Fourier transform infrared (FT-IR) absorbance spectroscopy and identified other sites and chemical mechanisms of bacteria injury leading to the VBNC state and cell death [205]. Upon exposure to low doses of chlorine (0.3 and 1 mg/L), chlorine-injured and dead bacterial cells retained the Fourier-transformed spectral properties of uninjured or live cells in the region of C-O-C stretching vibrations of polysaccharides, indicative of the cell wall peptidoglycan layer and lipopolysaccharide outer leaflet. The investigators concluded that extensive bacterial membrane damage is not a key factor in the inactivation of bacteria by chlorine, which is consistent with previous reports by others. Instead, chlorine caused changes in the spectral features of bacterial ester functional groups of lipids, structural proteins and nucleic acids, with apparent denaturation reflected between 1800 and 1300 cm (−1) for injured bacterial cells. In other studies of chlorine effects on bacteria, the role of reactive oxygen species has been cited as a contributing factor to injury and death. Tandon et al. reported that *E. coli* and *Enterococcus faecalis* exposed to 0.6 and 0.9 μg mL (−1) free chlorine in water were detected better when cultured under conditions that neutralized reactive oxygen species (ROS) using sodium pyruvate and anaerobic incubation [206]. The enumeration of chlorine-injured *Escherichia coli* and *Enterococcus faecalis* is enhanced under conditions where ROS are neutralized. In studies to elucidate dominant mechanisms of bacteria (*E. coli*) inactivation by ozone, chlorine dioxide, free chlorine and UV irradiation in water, inactivation resulted in protein release, lipid peroxidation, cell permeability changes, damages in intracellular enzymes and morphological changes [207]. Cell surface damage was greater with the strong oxidant ozone, while damage in inner cell components was greater with the weaker oxidant free chlorine. Chlorine dioxide inactivation mechanisms were intermediate between these two disinfectants. The authors concluded that the mechanisms of cell inactivation are primarily related to the reactivity of the chemical disinfectant. However, cell inactivation by UV radiation showed no measurable changes with the analytical methods employed.

There is evidence that copper injures *E. coli* cells by catalyzing the formation of reactive oxygen species, like hydroxyl radicals. The radicals produce oxidative damage near the unsaturated bonds of fatty acid chain segments of the phospholipid molecules which compose cell membranes [208]. The resulting structural disruption of membrane integrity results in numerous secondary effects, such as damage to membrane-associated oxidative respiratory enzymes [209]. Silver interfered with the synthesis of a ribosome-associated protein, subsequently inhibiting the synthesis of enzymes associated with glucose metabolism and other energy-producing processes [210]. The relationships between the proposed mecha-

nisms of bacterial damage and loss of culturability of the aforementioned studies to the VBNC phenomenon remain unclear. Overall, it is important that POU/HWT technology performance evaluation studies and their testing procedures address the well-documented evidence that bacterial physiological states, injury phenomena and the possible VBNC state will influence the extent to which test bacteria will be detected and reliably quantified depending on what culture-based assay methods, conditions and media are employed. These phenomena concerning the physiological states of bacteria cells are especially relevant to performance evaluation of POU treatment technologies based on disinfection (inactivation) of the microbes as opposed to their physical removal. For POU/HWT treatment technologies based on physical removal of the microbes, non-culture-based detection methods such as microscopy may be suitable to quantify the test microbes in performance evaluation studies.

11.4. Light-Induced Injury and Repair in Enteric Microbes

The role of light as a factor in inducing injury and as a factor in repair of injury by bacteria and possibly other cellular microbes in water is of importance to POU/HWT technology performance evaluation. This is because the possible positive and negative effects of light exposure during POU/HWT technology testing on the response of the test microbes is often not considered or accounted for, unless the POU/HWT technology being tested directly involves solar or UV lamp radiation as the disinfectant. Light in the ultraviolet wavelength range, originating naturally from the sun or artificially from germicidal lamps, can cause lethal or nonlethal injury to viruses, bacteria and protozoan cysts [211]. Short-wave UV light emitted by monochromatic low-pressure germicidal UV lamps at 254 nanometers, which is close to the wavelength most efficiently absorbed by DNA, exerts its effect primarily by causing formation of pyrimidine dimers on adjacent nucleotide bases of a DNA or RNA strand. This causes disruption of the hydrogen bonds between complementary nucleic acid strands, preventing normal replication. Longer wave UV and near-UV radiation in the 280 to 600 nm range, produced by sunlight and by medium-pressure germicidal lamps, causes damage and inactivation of microbes primarily by formation of highly reactive oxygen radicals like hydrogen peroxide, which react with and damage DNA, RNA and proteins.

Enteroviruses and RNA coliphage viruses, like other RNA viruses, possess an RNA-dependent RNA polymerase enzyme which replicates the virus genome. This enzyme possesses an unusually high error rate, inserting an incorrect nucleotide base as often as every 1500 bases [212]. The high error frequency, while yielding a large number of non-infectious progeny viruses, also permits the repair of damaged RNA. Single base repairs may occur as the result of a simple random base mutation during replication at the appropriate location. Investigators have uncovered impressive RNA repair capabilities through recombination, in which the viral RNA polymerase stops copying one segment and switches to another strand, linking them together. In one study, investigators removed 19 nucleotides from a critical region of RNA of coliphage MS2, which included two hairpin turns, a secondary structure element that is required for binding to a ribosome and subsequent synthesis of the major virus coat protein [213]. By random recombination, one viable mutant MS2 was formed by the insertion of an 18-nucleotide segment that restored the secondary hairpin structure, while a second mutant regained viability by excising 4 nucleotides, and rebuilding one of the hairpin loops, which also permitted successful binding of the rearranged RNA strand to the ribosome. Another study demonstrated that poliovirus rendered nonviable by removal of essential RNA segments could undergo recombination and recovery of infectivity in the absence of a functional viral RNA polymerase, suggesting that host cell processes were involved [214].

While these studies demonstrate that both pathogenic enteric viruses as well as coliphage indicator viruses can repair light-mediated damage, the repair process takes place in the course of replication within the host cell and would be difficult to be quantified or rendered more efficient by the analyst during assay of samples treated by POU technologies.

Bacteria demonstrate two processes, photoreactivation and dark repair, which can efficiently repair damage caused by sunlight or UV light produced by germicidal lamps. Dark repair is a process which does not require light energy. Dark repair involves a set of about a dozen proteins, which repair DNA by removing the segment of damaged nucleotides, then resynthesizing the excised DNA using the complementary DNA strand as the template. Photoreactivation of bacteria exposed to 254 nm UV radiation requires a few hours of exposure of injured bacteria cells to near-UV light in the 310 to 480 nm range to provide energy for the repair process. A bacterial enzyme, photolyase, eliminates nucleotide dimers. The rate of light repair is less dependent on the intensity of the light than on the exposure time, indicating that it is a process rather than an instantaneous chemical reaction. Exposure to fluorescent light for one to two hours at 37 °C or two to four hours at room temperature resulted in dramatic improvements of *E. coli* [215] and *Streptococcus faecalis* [115] recovery by 99.9% and 99%, respectively. To offset photoreactivation, the dose of 254 nm UV light required for 3-log inactivation of enteric bacteria would have to be doubled. Because light and dark repair may present challenges for the evaluation of POU technologies, manufacturers should provide detailed instructions on the dark storage or light exposure of water samples if these mechanisms are to be considered in the overall efficacy of the technology. However, unless light and dark repair reactions are directly related to the function of a POU device and necessary for regular use, HWT testing need not necessarily investigate these parameters.

When medium-pressure UV lamps, which emit a wider range of UV wavelengths, often at higher intensity, are used for disinfection of water, photoreactivation fails to occur [216]. Further investigation revealed that the photolyase enzyme was no longer functional [215]. Because the broader spectrum medium-pressure UV lamps produce reactive oxygen species that can damage proteins as well as DNA, photoreactivation can be halted.

These findings from disinfection studies on UV radiation suggest that the photoreactivation repair phenomenon may be a significant factor when low-pressure UV lamps or lamps emitting primarily at 254 nm are used in POU disinfection systems but may not be significant when wider spectrum lamps with higher intensity are employed. It would be prudent and possible to test initial product water samples treated by POU UV disinfection with and without photo-repair to determine if significant photoreactivation was occurring.

Two studies investigated photoreactivation and dark repair of *Cryptosporidium parvum* oocysts following exposure to UV light. One investigator found evidence that 30% to 60% of pyrimidine dimers were repaired during both light and dark repair, but the oocysts were not infective to mice [217]. A later study confirmed that UV-irradiated oocysts did not regain infectivity following light or dark repair conditions [35].

The role of light and its direct and indirect effects on microbe survival or inactivation also needs to be considered for a number of other water treatment technologies that involve Fenton and photo-Fenton reactions, such as reactions based on the use of hydrogen peroxide, hydrogen peroxide plus iron (soluble and oxide particles), titanium dioxide, or other metal oxide particles having antimicrobial oxidative reactivity that is light-mediated by Fenton reactions [218]. If such water treatment technologies based on potentially light-mediated advanced oxidation processes are used for POU/HWT, it is advisable and probably necessary that the role of light be considered and probably controlled in technology performance evaluation testing [203,219,220]. Possible ways to control such light-induced effects include conducting experiments in dark environments, under alternative light sources, or using opaque water vessels in which to do performance evaluation testing.

12. Culture Methods for Injured Bacteria

Culture media which could selectively grow pathogenic and other enteric bacteria were developed primarily for use with clinical samples analyzed in biomedical laboratories. These samples, often collected directly from human hosts, may contain a wide array of

viable and culturable bacteria, often at high concentrations, which are probably uninjured and likely to be actively growing and dividing. As the significance of the phenomenon of bacterial injury and repair in environmental and food samples became recognized, typical culture-based isolation and quantification procedures of biomedical laboratories were modified in such a way as to allow the recovery and quantification of that fraction of the bacterial population, which is injured, but still viable and potentially culturable. These bacterial resuscitation procedures usually involve an initial repair period under moderate incubation temperatures using nonselective media before transfer to more selective incubation conditions and selective/differential culture media to detect and quantify the specific target bacteria of interest. During the resuscitation period, injured bacteria repair cellular damage and regain the ability to grow and multiply in the presence of selective culture media components and elevated incubation temperatures.

Given the likelihood that a fraction of the challenge bacteria passing through a POU/HWT device may be injured by the microbial reduction treatment process being utilized, it is important that the procedures used to culture and quantify the challenge bacteria minimize further injury and enhance repair and subsequent detection of injured bacterial cells.

12.1. Sample Handling

An important consideration in the process of microbial enumeration in samples collected in the course of POU/HWT systems testing is the handling of the water samples during the period before the samples are analyzed, in order to minimize the loss or further injury of the target microbes. Sodium thiosulfate or an alternative reducing agent must be added to samples potentially containing oxidizing agents such as chlorine to neutralize oxidant residuals [220]. Disodium Ethylenediaminetetraacetic acid (EDTA) must be added to samples containing potentially toxic concentrations of multivalent, cationic antimicrobial metals such as copper to chelate the metal ions. Other neutralizers such as thioglycolate are recommended to neutralize monovalent cationic metal ions such as silver. Samples should be analyzed as soon as possible following collection, and should be stored at an appropriate temperature, usually 4 °C, until analysis begins. Samples should be diluted either immediately or before analysis using a chilled osmotically balanced buffered diluent, preferably containing a low concentration of proteinaceous or other stabilizing and protective materials such as peptones, antioxidants or other agents that can provide protection and stabilization against osmotic, oxidative and other sources of injury and starvation [221]. Molten agar which is to be mixed with a water sample should be tempered to no higher than 45 to 48 °C to avoid killing or further damaging bacteria cells, and samples should not be combined with molten agar at these temperatures until immediately before plating.

12.2. Incubation Temperature Conditions

As previously indicated, fecal indicator bacteria, such as thermotolerant (fecal) coliforms and *E. coli*, are frequently used as test organisms for evaluating the performance of POU/HWT systems. Thermotolerant or fecal coliforms are defined as coliform bacteria which can ferment lactose and produce gas at 44.5 °C. Early evaluations of the protocol for the enumeration of thermotolerant/fecal coliform bacteria in water by the membrane filtration technique found that a considerable fraction of the population was not detected if the filters were incubated directly at 44.5 °C. Presumably, this lack of growth was due to the inability of injured bacterial cells to grow at the elevated temperature, which is much higher than that of the normal intestinal habitat of fecal bacteria [222,223]. The operational definition of thermotolerant/fecal coliforms requires incubation at 44 to 44.5 °C in the presence of lactose for at least 18 h. However, researchers discovered that incubation of the membrane filters for an initial 2 to 4 h period at 35 to 37 °C, much closer to the optimal temperature for a fecal microbe in the intestinal tract, and subsequent transfer to 44.5 °C for the 18–22 h needed to achieve the required selectivity and growth of thermotolerant

coliforms, resulted in substantially higher recovery. Resuscitation of enteric bacteria is temperature-dependent, occurring at an increasing rate at temperatures of 15 °C and above, and taking place most efficiently at 35–37 °C [185]. An incubation temperature above 40 °C will kill injured thermotolerant/fecal coliform cells. The duration of the initial 35 °C incubation is limited in part by the potential appearance of nonfecal coliform colonies if the samples are incubated too long at the lower nonselective temperature [118]. This two-step incubation temperature process was also validated for use with the Colilert defined substrate medium for the detection of injured thermotolerant/fecal coliform bacteria and *E. coli* [224,225]. In other variations of these two-step procedures, a non-selective medium was used during the lower temperature incubation period, and the membrane filters were transferred to or overlaid or underlaid with a selective medium for the 44.5 °C incubation period. Injured thermotolerant/fecal coliform bacteria repair themselves during the initial 35 °C incubation period to the extent that they can then ferment lactose and multiply at 44.5 °C. The two-step incubation procedure has been widely adopted for enumeration of thermotolerant/fecal coliform bacteria in water and wastewater. A section discussing enumeration of stressed bacteria in recent editions of Standard Methods for the Examination of Water and Wastewater proposed two-step temperature incubation methods using Membrane Fecal Coliform (M-FC) agar, m-T7 agar or other culture media [226].

12.3. Culture Media

Culture media and enumeration methods designed to optimize the recovery of injured cells and spores of enteric indicator and pathogenic bacteria have been extensively reviewed [174,184–187,227]. Quantification formats include membrane filtration and then colony enumeration on solidified (e.g., agar) media quantal, liquid (e.g., broth) multiple tube most probable number (MPN) formats [220], including proprietary multi-volume, multi-well sample container formats like the IDEXX Colilert Quantitray system, for larger water sample volumes. Smaller sample volume enumerative repair procedures utilizing solid agar media in petri dishes for colony growth and counting include spread plates, pour plates and combinations of a nonselective agar medium either overlaid or underlaid by a selective agar either before or after the initial repair incubation period [227].

Different species of bacteria have different nutritional requirements and can experience variable degrees of multiple forms of structural, physiological and metabolic injury as a result of exposure to multiple kinds of deleterious environmental conditions. Because injury can be induced or exaggerated by culture conditions, researchers have investigated and proposed a wide variety of culture media formulations and culture conditions to enhance recovery of injured bacteria.

Bacteria require sources of carbon, nitrogen, essential vitamins and minerals to synthesize the energy-storing compound adenosine triphosphate (ATP) and to synthesize proteins, lipids, carbohydrates, nucleic acids and other critical structural and functional molecules. Consequently, both nonselective and selective culture media formulated to support the growth and multiplication of enteric bacteria must contain nutrients that supply these needs. Enzymatic digests of plant or animal tissue and milk protein (casein) are commonly used. Tryptone is an enzymatic digest of casein. It is a rich source of nitrogen, amino acids and vitamins. Tryptose and other peptones are enzymatic digests of a variety of proteins containing higher molecular weight peptides and longer chains of amino acids, which are used to support the growth of more fastidious bacteria. Beef extract is a more gently processed infusion of beef dried to a powder that provides amino acids, peptides, vitamins and minerals. Proteose peptone is an enzymatic digest of vegetable protein which supplies higher molecular weight peptide molecules. Yeast extract, the water-soluble fraction of autolyzed yeast cells, provides high concentrations of the B complex vitamins which stimulate bacterial growth. Defined substrate media contain specific chemicals as nutrients, stabilizing buffering ions and a specific nitrogen source such as M9 salts minimum broth containing only a carbohydrate, such as glucose, as well as magnesium sulfate and calcium chloride. Other defined substrate media such as IDEXX Colilert contains only ammonium

sulfate and three essential amino acids as nitrogen sources in addition to specific chromogenic and fluorogenic Beta-D-galactoside and -glucuronide defined substrates as carbon sources to support the growth of the target bacteria, coliforms and *E. coli*, respectively [224]. Most culture media formulations contain sodium chloride to provide balanced osmotic conditions in the medium. Potassium phosphate salts are often included to provide pH buffering capacity. Indicators of pH like neutral red and bromo cresol purple may be added to give a particular identifying and differential color to colonies of enteric bacteria which have fermented lactose, aniline blue to detect bacteria production of polysaccharides, or basic fuchsin as a growth inhibitor of non-target Gram-positive bacteria.

Because the complex nutrient sources listed above contain low concentrations of simple carbohydrates which can be metabolized for energy production, additional nutrients are usually provided. Soytone is a complex enzymatic digest processed from soybeans that contains relatively high concentrations of carbohydrates. Media formulated to enhance the growth of coliform bacteria such as lauryl tryptose broth, M-endo LES medium, EC medium, lactose broth, MacConkey agar, M-FC agar and MI agar contain lactose, because the ability to ferment lactose defines this group of bacteria. The presence of lactose as the only significant carbohydrate provides a considerable degree of selectivity to these culture media. In addition to lactose, MI agar contains indoxyl-ß-D-glucuronide, a substrate which is metabolized by an enzyme specific to *E. coli*, ß-D-glucuronidase to a blue-colored product, plus a second substrate, 4-methylumbelliferyl-ß-D-galactopyranoside, which can be metabolized to a fluorescent product in the presence of the enzyme ß-galactosidase, which is specific to total coliform bacteria. The defined substrate Colilert medium contains only two metabolizable compounds, methylumbelliferyl-ß-D-glucuronide, which is metabolized by only *E. coli* to a fluorescent hydrolysis product using the ß-glucuronidase enzyme specific to that microbe, and orthonitrophenyl galactoside, which is metabolized to a yellow-colored product only by total coliform bacteria utilizing the ß-galactosidase enzyme specific to members of that group. The use of chromogenic substrates has also been incorporated into agar-based media for use in water; examples include: CHROMagar, Rainbow Agar and HiCrome Agar [228].

Selective and differential media often contain certain toxic agents to inhibit non-target microbes and, in this respect, differ from nonselective bacterial culture media. The class of agents most commonly used in media formulated to select for enteric bacteria is bile or its derivatives. Bile is a complex mixture of bile acids, phospholipids, cholesterol and other substances which is produced in the liver, where it functions in the digestion and absorption of fats in the intestine by emulsification and solubilization of lipids. In high concentrations, bile can kill bacteria by destroying cell membranes [229]. At lower concentrations, bile can penetrate into bacterial cells and disrupt cell membrane-associated activities. Like several other bactericidal substances, bile can also cause cell injury or death by promoting the formation of oxygen free radicals inside bacterial cells which can damage DNA and proteins. Virtually all bacteriological culture media designed to selectively grow enteric bacteria contain bile salts, oxgall, which is a purified form of bovine bile, sodium deoxycholate, the salt form of one of the bile acids, or sodium lauryl sulfate, an anionic surfactant which can also emulsify lipids.

Bacteria normally habiting the intestines of warm-blooded animals possess cell membranes and membrane-associated proteins which have evolved to protect the cells from the actions of bile [229]. Gram-negative enteric bacteria are more resistant to bile than Gram-positive bacteria. Bacterial cells which have suffered injury from detrimental environmental conditions or from a POU/HWT drinking water treatment process, and have already accumulated membrane or protein damage, may subsequently experience lethal damage if exposed to bile in the culture medium if the injured cells are not allowed the time and conditions necessary to resuscitate.

Like bile, crystal violet is a selective agent which inhibits the growth of Gram-positive bacteria, as is cefsulodin, which is an antibiotic that inhibits Gram-positive bacteria and some non-coliform Gram-negative bacteria. Cycloserine and polymixin B sulfate are

antibiotics used in some media designed to enumerate *Clostridium perfringens* to suppress non-*Clostridium* species of bacteria. Sodium azide is a component of m-Enterococcus agar, which suppresses Gram-negative bacteria. These selectively toxic compounds can also inhibit the growth of the target organism if the cells have accumulated excessive damage that has not yet been repaired.

No specific culture media that can both selectively provide optimal enumeration of any target enteric bacterial species or group while at the same time permitting efficient resuscitation of injured bacterial cells are available. One approach to this situation is to formulate a culture medium containing a minimal number of selective components with lower toxicity in order to minimize inhibition of injured cells of the target microbe. This approach was taken for the formulation of M-T7 agar designed to enumerate total and fecal coliforms injured by chlorine in drinking water [230]. Standard Methods for the Examination of Water and Wastewater suggests a strategy for enumeration of injured fecal coliform bacteria involving the step-by-step deletion of selective agents from a culture medium, while recommending that the identities of presumptive positive colonies be verified by biochemical testing [226]. A second approach, common to both broth culture method and membrane filtration methods, is to first recover microbes using a non-selective medium followed by a selective one. Microbes commonly detected and quantified using this approach include *Salmonella* spp. and *Clostridium perfringens*. An alternative optimal approach is the formulation of a defined substrate medium like Colilert, which uses specific carbon sources that can be metabolized only by the target microbes as its primary source of selectivity, as well as the provision of growth nutrients, thereby avoiding the need for any toxic selective components.

12.4. Bacterial Injury and Repair in POU Testing Protocols

In summary, the phenomenon of injured enteric bacteria cells is expected to be associated with both the testing of POU/HWT technology systems in the laboratory and the utilization of POU/HWT systems in the real world. Source waters for household POU/HWT systems will most likely contain populations of enteric bacteria that have been injured by deleterious environmental conditions. Therefore, it may be appropriate for seed stocks of bacteria used to challenge POU/HWT systems in the laboratory to likewise contain relatively high proportions of stressed bacteria. In addition, some of the POU/HWT systems, especially those based on chemical or physical disinfection, will themselves produce additional injured bacteria in the product water. Consequently, it may be advisable for testing protocols to include sample handling procedures which limit further stress to injured cells. These injury repair and resuscitation measures include incubation protocols with assay methods that provide sufficient resuscitation time at moderate temperatures and use culture media containing minimal toxic selective agents as well as protective chemical agents, primarily reducing agents, such as oxyrase, catalase, ascorbic acid and pyruvate, that promote resuscitation of injured bacteria [231,232]. The selection of quantification methods for injured bacteria that have been approved or recommended by established sources such as the Environmental Protection Agency, the American Public Health Association or other authoritative agencies would avoid the need for extensive validation of new or alternative customized assay procedures and materials. Of the media and culture conditions available from these resources, care should be taken to select the media and culture conditions identified as preferred for the recovery and detection of injured or stressed organisms.

The 1987 EPA Protocol explicitly states that a "research need" like distinguishing between dead and injured microbes is inappropriate "at this time" for inclusion in a standard protocol. This suggests that the phenomenon of microbial injury and repair was not sufficiently established or documented at the time nor were remedies for such injury well established so as to be a part of a protocol formulated for widespread acceptance and use. The EPA protocol does later state, however, that the analytical methods should be updated as improved procedures are developed.

As a consequence of this position, the 1987 EPA guide standard protocol recommends standard methods to enumerate coliform bacteria challenge microbes using selective media like mFC, which contains bile salts, or m-Endo medium, which contains sodium desoxycholate and sodium lauryl sulfate as selective agents. Additionally, no alterations in incubation temperatures are specified or suggested to encourage the resuscitation of injured bacteria. Therefore, this protocol specifies the use of media and methods that are well-documented as inferior in recovering injured bacteria.

The NSF Protocol P231 makes no alterations to these procedures. Likewise, the NSF/ANSI Standard 55 written for UV systems made no mention of cell injury or procedural changes allowing for light or dark repair by injured microbes. The Chinese protocol did not address the concept of bacterial injury and repair.

The NSF Military Protocol P248 also did not raise and address the issue of bacterial injury or include recommendations for assay procedures to allow bacterial resuscitation. The Military Protocol recommended the use of a selective medium, m-Endo agar, which, as noted above, contains a number of injurious selective agents, to enumerate the challenge coliform bacteria.

In the Evaluating Household Water Treatment Options document of WHO, mention is made that bacterial cells may be injured during exposure to environmental conditions in surface water, and that the degree of injury may influence their survival in the course of treatment by a household POU/HWT device. However, no specific recommendations are made about choosing bacterial quantification procedures which enhance the recovery of injured bacteria.

The Chinese Ministry of Health protocol does not address the issue of bacterial injury and repair. The procedure calls for enumeration of *E. coli* using a selective Endo agar, which contains chemical agents that would inhibit bacterial repair. The Brazilian and Mexican protocols likewise do not mention bacterial injury and repair, but both suggest the use of a wide range of bacterial enumeration methods, which include both selective and injury repair culture methods.

Overall, the extent to which the various protocols and guidance documents for performance evaluation of POU/HWT technologies address and account for the resuscitation and repair of injured bacteria is variable and inconsistent and overall, they are either inadequate or silent on the issue. Most make no mention of and do not directly address injury at all. Most specify or recommend the use of media and growth conditions that are inadequate or inappropriate for repair and recovery of injured bacteria. A few protocols acknowledge the issue but provide no specific guidance, recommendations or procedures to specifically address the recovery of injured bacteria by the choice of growth media and recommended or specified incubation conditions.

13. Specified Test Water Matrices

Currently, there are four documents that detail test waters utilized for the examination of POU/HWT technologies. The three institutions that sponsor these documents are the WHO with their "Evaluating Household Water Treatment Options: Health-Based Targets and Microbiological Performance Specifications", the US EPA, with the "Guide Standard and Protocol for Testing Microbiological Water Purifiers", the NSF-International (formerly National Sanitation Foundation) with methods of NSF/ANSI Standards 53, 55, 58, 62 and 244, "NSF Standard for Drinking Water Treatment Units," method P248 "Military Operations Microbiological Water Purifiers" and NSF P231 Microbiological Water Purifiers. When considering these protocols and comparing their test water specifications, it is important to recognize and consider the extent to which there is cognizance, consideration and modeling or mimicking of real-world waters with respect to possible presence of fecal contamination, as well as other contaminants that may be present in environmental settings. An important goal in evaluating POU/HWT devices is to challenge them with waters that may be encountered in the settings in which they may be used to produce household drinking water for the intended use based on duration of time or cumulative water volume

treated. Therefore, the water for challenging the technologies should be representative of real-world waters the device is intended to treat for the duration of time or total volume of use.

13.1. WHO Evaluating Household Water Treatment Options

The WHO Recommendation document identifies two different types of test waters for the verifications of POU/HWT technologies. The first test water is suggested to be of high quality, as either groundwater, surface water, harvested rainwater or other water with no disinfectant residual, with a turbidity of less than 5 NTU, a pH of between 7.0 and 9.0 and a temperature of 20 ± 5 °C. The second test water should also be a high-quality groundwater, surface water, or rainwater without a disinfectant residual and amended with 20% by volume primary wastewater effluent or 1% by volume untreated raw sewage, which can either be sterilized or pasteurized. This water should have a turbidity of greater than 30 NTU, a pH between 6.0 and 10.0 and a temperature of 4.0 ± 1 °C. This protocol also suggests that at least 20 L per day should be the challenge volume used in laboratory challenge testing or verification, unless otherwise specified by manufacturer's recommendations [7].

In terms of altering the characteristics of the test waters to increase turbidity, the WHO document suggests the use of the air cleaner (AC) fine test dust originally manufactured by the AC Spark Plug Division of General Motors and specified in the US EPA GS protocol, or if difficult to obtain, the use of finely sized clay particles. To increase the pH of test waters, the use of inorganic acids or bases is suggested, and examples include hydrochloric acid and sodium hydroxide. This suggested testing method does not specify test microbes, but rather allows consideration of microbes specific to the area where a POU/HWT technology would be used. This allows the treatment device to be examined specifically for its ability to remove or inactivate particular pathogens of health concern in certain geographic areas [9].

13.2. EPA Guide Standard Protocol

In the US EPA GS, there are 5 suggested test waters with specific chemical and physical properties. Test water #1 is designated as general test water and is intended for use during the non-microbial challenge phase of the examination of test unit performance. This water is to be free of any residual disinfectant, have a pH between 6.5 and 8.5, a Total Organic Carbon (TOC) concentration between 0.1 and 5.0 mg/L, a turbidity of 0.1–5 NTU, a temperature of 20 ± 5 °C and a Total Dissolved Solids (TDS) concentration of 50–500 mg/L. Test water #2 is intended for the microbial challenge phase of testing for treatment units employing halogen disinfection. The challenge water is to have no residual disinfectant, a pH of 9.0 ± 0.2 (or for iodine-based units, a pH of 5.0 ± 0.2), a TOC of at least 10 mg/L, a turbidity of at least 30 NTU, a temperature of 4 ± 0.1 °C and a TDS value of 1500 ± 150 mg/L.

Test water #3 is also intended for the challenge phase of testing, but for ceramic filter units with or without silver particles. This water will have no residual disinfectant, a pH of 9.0 ± 0.2, a TOC level of 10 mg/L or greater, a turbidity of at least 30 NTU, a temperature of 4 ± 0.1 °C and a TDS of 1500 ± 150 mg/L. Test water #4, again, is intended for the microbial challenge phase of testing but for UV units. This water will have no residual disinfectant, a pH of 6.5–8.5, a TOC of at least 10 mg/L, a turbidity of at least 30 NTU, a temperature of 4 ± 0.1 °C, TDS of 1500 ± 150 mg/L as well as a color UV absorption level just below the trigger point of the warning alarm on the UV unit. Test water #5 is the final test water designed for the stressed challenge phase of examining POU/HWT technology unit performance. This water is designed as a leaching test of units containing silver, and it will have no residual disinfectant, a pH of 5.0 ± 0.2, a TOC of approximately 1.0 mg/L, a turbidity of 0.1–5 NTU, a temperature of 20 ± 5° C and a TDS of 100 mg/L (US EPA, 1987).

For adjusting the chemical properties of the test waters, the recommended materials are inorganic acids or bases for pH, humic acids for TOC, AC Fine Test Dust for turbidity

(the same as recommended by the WHO and US EPA GS Protocol), sea salts from Sigma Chemical CO for TDS and p-hydroxybenzoic acid for color UV absorption adjustment.

The US EPA also suggests specific microbes for the examination of performance of POU/HWT technologies. The suggested bacteria are *Klebsiella terrigena*, which should be spiked at a concentration of 10^7/100 mL. The recommended viruses are either Poliovirus 1 or Rotavirus SA11, both spiked at concentrations of 1×10^7/L. The protozoan recommendation is *Giardia* cysts of either of *Giardia muris* or *Giardia lamblia* spiked at a concentration of 10^6/L. However, the US EPA also allows the use of spheres or particles of 4–6 microns in diameter as an alternative to *Giardia* cysts for POU treatment units based on filtration [22].

13.3. NSF Standard 244

NSF-International has also suggested general test water for microbiological performance evaluations of POU/HWT technologies, which may be altered for specific testing conditions and technology applications. In method 244, the NSF recommends that the water to be used for general testing first be treated by reverse osmosis and then deionization, resulting in water having no detectable residual disinfectant. This water is to be adjusted to a hardness of 50 ± 10 mg/L, a pH of 7.5 ± 0.25, a temperature of 20 ± 2.5 °C and a TOC of 3.0 ± 1.0 mg/L. The protocol suggests that the hardness be adjusted by adding $CaCl_2$ and $MgSO_4$, TDS be adjusted using a NaCl solution, tannic acid to adjust the TOC and a sodium bicarbonate solution to adjust the alkalinity [18].

Method 244 identifies the use of the challenge bacteria, *Raoultella terrigena. R. terrigena* is to be spiked into general test water at concentrations of greater than or equal to 5×10^7 cfu/100 mL with an average maximum of 5×10^8 cfu/100 mL. This method indicates that the spiked general challenge water be analyzed for the bacteria at the beginning of each test, at each sampling point and at the end of use of the test waters prepared in a batch system. The suggested challenge viruses are the coliphages fr and MS2, both spiked into general challenge water to at least 5×10^6 plaque forming units (pfu)/100 mL, with an average maximum of 5×10^7 pfu/100 mL. There is no protozoan challenge as the Standard indicates that reverse osmosis (RO) systems passing tests for both viruses and bacteria can make a protozoan cyst performance claim.

13.4. NSF Protocol P248

The NSF-International document for evaluation of POU/HWT technologies for emergency military operations, Protocol P248, also suggests 5 test waters for varying types and conditions of the specific POU technologies. Test water 1 is intended as a general test water, with a chlorine level of less than 0.1 mg/L, a pH of 7.0 ± 0.5, a TOC of 0.1–2 mg/L, a turbidity of less than 1 NTU, a temperature of 20 ± 5 °C, a TDS of 50–500 mg/L, an alkalinity as $CaCO_3$ 100 ± 20 mg/L and a background bacteria level of greater than 10^3 cfu per 100 mL. Test water 2 is a halogen challenge test water that should have a chlorine level of less than 0.1 mg/L, pH of 9.0 ± 0.2 (pH of 5.0 ± 0.2 for iodine-based units), TOC of 10–15 mg/L, turbidity of 30–50 NTU, temperature of 4 ± 1 °C, TDS of 1500–300 mg/L, alkalinity as $CaCO_3$ 100 ± 20 mg/L and background bacteria level of greater than 10^3 cfu per 100 mL [14].

Test water 3 is a filter challenge test water and should have a chlorine level of less than 0.1 mg/L, pH of 9.0 ± 0.2, TOC of 10–15 mg/L, turbidity 30–50 NTU, temperature of 4 ± 1 °C, TDS of 1500–300 mg/L, alkalinity as $CaCO_3$ 100 ± 20 mg/L and background bacteria level of greater than 103 cfu per 100 mL. Test water 4 is intended as a UV challenge water and should also have a chlorine level of less than 0.1 mg/L, a pH of 7.0 ± 0.5, a TOC of 10–15 mg/L, a turbidity 30–50 NTU, a temperature of 4 ± 1 °C, a TDS of 1500–300 mg/L, an alkalinity as $CaCO_3$ 100 ± 20 mg/L, a background bacteria level of greater than 10^3 cfu per 100 mL and a measured ultraviolet light transmittance at 254 nm. Test water 5 is intended as a silver leaching test water and should have a chlorine level of less than 0.1 mg/L, a pH of 5.0 ± 0.2, a TOC of 0.1–2 mg/L, a turbidity of less than 1 NTU, a temperature of 20 ± 5 °C, a TDS of 25–100 mg/L and an alkalinity as $CaCO_3$ of 100 ± 20 mg/L [14].

Method NSF 248 also suggests using inorganic acids or bases to adjust pH, tannic acid to provide TOC, fine test dust according to ISO to provide turbidity, sodium chloride or sea salts from the Sigma Chemical Co to provide TDS levels and sodium bicarbonate to provide alkalinity. The suggested spiking microbes include bacteria, viruses and protozoan cysts, as specified in Supplementary Table S1. The recommended bacteria include *Escherichia coli, Raoultella terrigena* or *Bacillus atrophaeus* (spore form) at concentrations of at least 10^7/100 mL. The suggested test viruses are MS2 and fr coliphages spiked at least 10^7/L, while the recommended test protozoan parasite is *Cryptosporidium parvum* in the form of purified oocysts spiked at concentrations of greater than or equal to 5×10^4/L. *C. parvum* oocysts stocks, influent samples and effluent samples are to be processed and enumerated according to the membrane filtration/fluorescent microscopy protocol described in the most current version of ANSI/NSF Standard 53—"Drinking Water Treatment Units—Health Effects." For POU/HWT technologies that are solely non-mechanical filtration devices, the infectivity of the *C. oocyst* cyst stock must be determined by cell culture assay method and presumably, this assay must be used for challenge test influent and effluent samples as well.

There are considerable differences in the required or recommended test water qualities to be used for examining the performance of POU/HWT technologies in challenge experiments. The specified test waters of the different protocols were presumably designed to characterize the performance of different types of POU/HWT technologies yet be applicable to various modifications of the technologies and their use in different POU/HWT treatment situations and settings. However, it is important to note that many of the specified waters are not fully representative of environmental waters, as most do not have sewage or natural aquatic organic matter present in them and the primary source of turbidity is mineral clay particles and not biological or other organic particles. In addition, although it is suggested to test specific microbial indicators or pathogens, it is uncertain if these specified test microbes are representative of the important disease-causing pathogens likely to be present in source waters of specific geographic locations and at different ambient temperatures and seasons.

It is important for those developing and conducting testing programs and specific studies to choose test microbes that are representative of those present in real-word settings and their waters where the POU/HWT technology is to be used. However, only the WHO performance evaluation document suggests using microbes relevant to the area in which the technology will be used, as well as using sewage to introduce the organic constituents typically found in environmental source waters. The use of sewage recognizes and addresses the impacts of human waste as a reality and concern resulting from prevailing inadequacies in sanitation conditions and their impacts on source waters of drinking water. Likewise, the types and levels of TOC, turbidity and TDS as well as the pH levels of test waters also need to be representative of the waters of interest in the geographical regions and settings where the POU/HWT technology is to be used. Therefore, it is important to evaluate and characterize the water quality situation of the areas in which a POU/HWT technology will be used before designing and implementing challenge studies to evaluate its performance efficacy.

14. Point-of-Use System Testing Schedules

POU/HWT systems for drinking water production possess differing effective water treatment lifetimes. For some technologies, system performance may vary with the volume of water treated due to declines in functionality over time. For example, some filtration technologies have filter elements that decline in performance due to clogging or other phenomena, resulting in declines in flow rate and rate of water production that cannot be restored by cleaning and requiring eventual replacement of a filter element. It is important that the POU/HWT technology testing protocols are of sufficient duration in terms of use time or cumulative volume of water treated to account for changes in flow rate and possible changes in performance. Declines in filter flow rate may influence not only

microbial reduction performance but also user satisfaction and willingness to continue filter use [233].

Besides flow rate decline and eventual clogging, some of these filter elements may decline in microbial reduction performance efficacy over time and with increased volume of water filtered. This has been observed with certain ceramic filters [234]. Some chemical disinfection POU/HWT technologies may rely on disinfectant delivery elements, such as erosion chlorine elements or halogen ion exchange resins, that will eventually become exhausted and no longer deliver any disinfectant with increasing volume of water treated. For POU/HWT technologies that may decline in performance with increasing volume of water treated, the nature and extent of microbial reduction performance needs to be characterized, quantified and accounted for, based on the manufacturer's specifications and performance claims.

Therefore, it is necessary to establish a POU/HWT system testing plan and schedule for microbe and water quality composition challenges that reasonably model conditions faced by the POU/HWT technology system during its functional lifetime. Some types of POU/HWT systems have effective volume lifetimes set by the manufacturer and based on expected or claimed treated water volume, beyond which declining performance, such as reduced concentrations of delivered disinfectants, may compromise the product performance and treated water quality. It would be advisable for the testing protocol to acknowledge that some technologies have an advertised lifespan, exhaustible components, or automatic "shut-offs", once they have reached the end of their functional lifespan. Testing should take into consideration the maximum volume lifetime of the technology, or when a vital part of the technology loses the ability to deliver safe water.

Other types of systems, like the biosand filter and other biologically active filters, may have unspecified lifetimes extending for years once their biological activity is established and assuming they are periodically cleaned according to the provider's operating and maintenance instructions. However, an important consideration in performance evaluation scheduling and timing cycles for such biologically active filters concerns the amount of time it takes for such biological activity to be established (filter maturation or ripening) and, once established, how long filters can operate before the need for periodic filter cleaning to remove accumulated particulate and excessive biological growth impurities and restore declined flow rates. These cyclic factors can impact microbial reduction performance, with decreased microbial reductions prior to filter maturation and perhaps also after a periodic cleaning event. The time periods for filter maturation, the duration of filter run times before the need to clean and the influences of these processes on microbial reduction performance are likely to be related to the quality of the raw water, especially its content of biodegradable organic carbon and other required nutrients for effective biological activity, as well as its turbidity, which can contribute to filter clogging and declining flow rates that necessitate filter cleaning. Performance evaluation challenge studies should be designed to evaluate microbial reduction performance through these steps of initial start-up and maturation, typical operation at maturation eventually leading to declined flow rates and filter cleaning followed by subsequent re-establishment of maturation [235].

14.1. Testing Schedule Considerations

Adequately testing the wide variety of POU/HWT system designs in the laboratory within a reasonable timeframe, at an acceptable cost and with representative testing durations, including periodic challenge testing and testing "end-points", requires careful consideration and planning. Some testing protocols and standards specify that these conditions are to be selected based on the manufacturer's guidance for effective lifetime use, usually in terms of the volume of water which can be effectively treated before the technology must be serviced or components replaced. However, some protocols specify or recommend a more arbitrary test duration, often 14 days, for those POU/HWT devices like filter-based systems, such as the biosand filter, which have variable performance cycle times, durations and use conditions and are intended to have very long operational life-

times. Typically, the total test period is divided into a reasonable number of equal volume spaced fractions or percentages of treatment unit total volume life, often five fractions or percentages in number, to provide test points where the system is challenged with test microbes. In some protocols, influent water of a poorer quality is tested at some or all volume fractions, which can adversely affect the microbe reduction efficiency of the POU/HWT units being tested and represents a "worst case". The initial challenge test is conducted just after a set of replicate POU/HWT units have been conditioned or flushed according to the manufacturer's instructions.

Operational water quality measurements like disinfectant residuals, turbidity and pH are also measured in the influent and product water at these challenge times. In some test protocols, the operating intervals between challenge water test points use an easily provided relatively high-quality water, such as dechlorinated tap water, for up to 16 h per day in order to achieve the manufacturer's volume lifetime target within a reasonable time. Some testing protocols and schedules include a pair of 48 h stagnation points during which no water flows through the POU/HWT device, the first of these at the half-way volume mark, and the second near the end of the test run total volume. A stagnation period may be deleterious to the microbial removal efficiency of some types of POU/HWT systems based on filtration processes. However, prolonged idle times have been demonstrated to enhance the microbial reduction performance of the biosand filter [236]. Such enhanced microbial reduction performance of the biosand filter during a stagnation period may be a result of the prolonged contact time between the challenge test enteric microbes in the stagnant water with members of the established microbial community in the stagnant water and on the filtration media, which allows for antimicrobial processes such as predation by members of the filter microbial community or inactivation or biodegradation caused by antimicrobial chemicals such as extracellular proteolytic enzymes that are produced by the filter microbial community [236]. A prolonged stagnation period may be unrealistic or unreliable for actual field use of a POU/HWT device, as household members would likely produce treated water almost daily and perhaps more frequently, such that any beneficial effects resulting from prolonged idle times of water within the filter medium do not occur. However, if the manufacturer of such a biologically active filter specifies specific idle times, they should be included in performance evaluation studies.

14.2. POU Testing Protocol Schedules

The 1987 EPA Guide Standard and Protocol establishes a testing schedule in which triplicate POU/HWT treatment units are first conditioned or flushed according to the instructions of the manufacturer with higher quality aging water (general test water) not seeded with the challenge microbes. There are two test water phases: a general test water phase (unstressed) and a challenged test water phase (stressed). Following conditioning, the units are periodically challenged with test microbes at regular fractions of the lifetime of the units, with the test schedule and challenge points being technology-dependent and resulting in challenging and sampling at: start, 25% and 50% interval challenges with the general test water, and 60%, 75% and 100% microbe challenges for the challenge test water, or start, days 3 and 6 for the general test water and days 7, 8 and 10.5 for the challenge test water. The US EPA protocol also calls for microbe challenges following 48 h stagnation periods after the 50% and 75% intervals or following days 6 and 8. For all technologies, units are not operated continuously and thus are 'at rest' overnight. The lifetime of a POU/HWT device is defined as the total water volume treatment capacity specified by the manufacturer for systems using chemical disinfectants. A test period of 10.5 days is recommended for UV or filtration-based systems which may not have specified total water treatment volumes. Aging of the systems with the higher quality water is done for no longer than 16 h in a 24 h period. The maximum flow rate is that established by the manufacturer. Maintenance and component replacement are done according to the manufacturer's instructions. The NSF Protocol P231 follows the EPA GS protocol recommendations without alterations.

The NFS/ANSI Standard 55 for testing UV systems establishes a testing schedule on a temporal basis, as UV units possess less well-defined operational lifetimes. Microbial challenges of duplicate POU devices are carried out twice a day four hours apart on the start-up day, and on days 1, 2, 3, 4 and 7, with a 48 to 72 h stagnation period during days 5 and 6. There is a 16 h stagnation period at the end of each day.

The Chinese protocol tests duplicate systems. The protocol specifies using the manufacturer's guidance of the total water treatment capacity of the system as the lifetime of the system. That parameter is divided into four intervals, and challenge testing is conducted on start-up and when 25%, 50%, 75% and 100% of the total water treatment volume is reached. There is no mention of stagnation periods in the testing schedule. The Chinese Ministry of Health protocol follows the same testing schedule. There is no mention of testing replicate POU units.

The NSF P248 testing protocol for military use specifies testing triplicate units. The POU unit lifetime is based on the manufacturer's stated total volume of water which can be treated prior to replacement of functional treatment components. However, the test capacity is either 135 L based on a use pattern of 15 L per day for a product intended for individual use or is the volume accumulated after 10 days of flowing with an identified use pattern for larger, squad sized units. A higher quality general test water is used for aging and testing the system throughout the first half of the test process, and a specified worst-case challenge water is used during the final half of the test. Microbe challenges are conducted at start-up and at 25%, 50%, 60% and 75%, a second challenge following a 48 h stagnation period after the 75% interval and at the 100% interval. The initial start-up microbe challenge is to be conducted immediately after setting up the devices unless the manufacturer specifies that the systems need to be conditioned prior to use.

The Brazilian protocol calls for testing a POU/HWT device for bacterial removal only at startup and after 95% of the manufacturer's total water treatment volume capacity. There is no requirement for testing of replicate POU units.

The Mexican protocol for bacterial reduction specifies only a single sample collection interval at ten minutes after commencement of operation of the POU device. There is no requirement for the testing of replicate POU units.

15. Safe Storage and Handling of Treated Water

In previously reported field studies on household access to clean water, either for water from a community point source or following treatment by individual POU/HWT technologies, the household water may not be safe when consumed for a variety of reasons. A meta-analysis of 57 field studies that compared coliform bacteria concentrations in the source water with water stored in the home found that half of the studies reported significantly higher coliform bacteria counts in households than in source water [237]. Contamination and recontamination often occurred as a result of water stored in the home having contact with contaminated hands or drinking vessels that were infrequently or inadequately washed [238]. The use of uncovered wide-mouth water storage containers and the presence of children and domestic animals increased the possibility of contamination in the household-stored water. There was an absence of any residual disinfectant in many cases, as this was not believed by the consumers to be needed because the water was thought to be clean. Hence, residual disinfectant had been eliminated as a barrier to recontamination [239]. Encouragement of the use of capped, narrow-mouthed water storage containers was found to reduce microbial recontamination of household stored water [240,241].

Recommendations for changes in hygienic behavior "to keep clean water clean", at least in the short and medium term, were more acceptable to people if they could be shown direct evidence that they were at risk because water in their own household was contaminated, if they were informed their drinking water could be kept cleaner by making recommended water management changes and if they were trained to effectively

use and maintain POU/HWT devices and to protect the quality of their treated drinking water [242,243].

Testing of POU/HWT devices which possess an integral or separate water storage compartment as part of the system should include at least some testing to determine if challenge fecal microorganisms are present in the water of the storage vessel and/or are associated with biofilm on the walls of the storage vessel. Such testing is best carried out relatively late in the testing schedule, by which time biofilm formation may be expected to have occurred. Samples should be collected well after the most recent microbe challenge has occurred and the system has been thoroughly flushed with unseeded water. Treated water in the storage vessel or compartment could be sampled before and after scraping or swabbing its walls to dislodge biofilm-associated microbes. To examine the possibility that injured bacteria in the product water undergo metabolic repair during storage, water could be assayed for bacteria immediately after treatment and again after several additional hours of storage in a container or vessel which had recently been disinfected to eliminate biofilms as a confounding alternate source of challenge test bacteria.

Even if a POU system is not provided with a product water storage container, it is essential that the designers, manufacturers, providers and distributors of POU/HWT devices understand the need for proper product water storage, handling and dispensing as a critical barrier to maintain the microbial quality of the water treated by their device. Failure to protect the quality of the product water will most likely negate many of the microbial quality and potential health benefits associated with an otherwise effective POU/HWT system being used in the home. Members of the household need to be educated concerning proper storage and handling of treated water, and safe treated water storage containers must be readily available. Manufacturers and distributors of POU/HWT devices should either provide a safe storage vessel as part of the unit marketed or encourage consumers to additionally purchase and use a safe storage vessel or container.

Of the testing protocols reviewed, only the P248 military protocol raises the issue of post-treatment storage and handling of the POU/HWT system product water to prevent recontamination. The P248 protocol merely states that the treatment unit must be designed to allow filling and servicing of the unit without re-contaminating the product water or any surfaces that contact the product water. The WHO Evaluating Household Water Treatment Options document does advocate measures to minimize recontamination by the use of closed or narrow mouth storage containers and downward-facing spigot dispensers, but states that household water handling issues are otherwise not specifically addressed in the document. The intent of NSF Standards and Protocols and WHO Scheme evaluation is that if a product contains storage as a component of the system, this storage component will remain intact and be used in the evaluation of the device or system.

16. Operational Monitoring of Point-of-Use Systems during Testing

Analysis of POU/HWT system samples to determine the extent of reduction of challenge microorganisms in the product water usually requires at least one day of elapsed time to provide microbe concentration data from microbial challenge tests. This is because of the need to obtain results from culture-based microbial assays of challenge test samples. There may be certain physical or chemical parameters of the product water which can be measured while the system is in operation that provide near real-time indications of the performance of the POU/HWT system. The relevant non-microbial performance parameters to be measured are determined by the nature of the treatment technology being utilized by the POU/HWT device, but they could be turbidity for filtration technologies and disinfectant residual for chemical disinfection technologies, as examples.

Operational monitoring is useful not only during testing of POU/HWT systems in the laboratory but may also provide the only indications of the effectiveness of the device in producing safe drinking water to actual users of the POU product in their households. Some of these parameters can be perceived directly by the users with their own senses (e.g., elevated water temperature from boiling or pasteurization, visual clarity

of the water from filtration or the smell of chlorine from chlorine dosing), and these will be influential in forming their opinions concerning the acceptability and value of the POU/HWT device itself.

In order to provide an acceptable volume of suitable quality water for a household, a POU/HWT system must deliver product water at a reasonable flow rate. POU/HWT systems like biosand or ceramic filters or devices which utilize another type of filtration component such as a filtration cartridge, may exhibit a declining flow rate over time. A gradual but significant decline in flow rate may indicate to the user that cleaning or replacement of a component is needed to improve the flow rate. A sudden unexpected increase in the flow rate may indicate failure of a filtration component or "short-circuiting" in the system, which might allow passage of water through the system with inadequate treatment.

POU/HWT systems which utilize disinfecting chemicals to eliminate microbes often yield product water which contains residual concentrations of the disinfectants, unless the technology has an additional treatment step to remove the disinfectant from the water before it is to be consumed. The WHO provides health-based guideline values for these chemicals in drinking water [7]. While avoiding excessive and potentially toxic levels of chemicals in the product water, a POU/HWT device may need to maintain a level of residual disinfectant sufficient to render microbes non-infectious throughout the lifetime or service cycle of the device. Measurement of disinfectant concentrations in product water would provide evidence that both of these objectives are being met. Because a POU/HWT device may deliver an initial spike of disinfectant chemical in the initial period of water flow, and because the level of disinfectant may decline with use and over time, periodic monitoring should be conducted throughout the use cycle of the device and its estimated lifetime of use or replacement of elements that eventually become ineffective with continued use.

Direct correlations have been found between the turbidity levels of surface waters used as sources of drinking water and concentrations of *Giardia* cysts and *Cryptosporidium* oocysts [244]. Reduction of turbidity during drinking water treatment processes has been shown to be significantly correlated with removal of *Giardia* cysts and *Cryptosporidium* oocysts in both pilot [245] and full-scale [246] treatment facilities. Efforts to improve the quality of drinking water by more effective filtration through regulation [247] and through operator training [248] were predicated on turbidity reduction and associated removal of protozoan cysts and oocysts. Periodic measurements of the turbidity of both challenge water and product water during testing of a POU/HWT device may provide useful indications of the performance of the system. Furthermore, because the visible clarity of drinking water is often used by the consumer to judge its quality, the ability of a POU/HWT system to appreciably reduce turbidity may play an important role in the gaining and maintaining user acceptance of the POU device in the household [7].

In addition to or as an alternative to monitoring of flow rate, residual disinfectant concentrations and turbidity, other operational parameters specific to the POU/HWT technology of the system being tested may provide useful information concerning its performance. For example, intensity of a UV lamp may be indicative of its performance and effective delivery of the desired amount of UV radiation. Monitoring temperature may be indicative of the performance of a technology that relies on increasing temperature to kill microbes in water, such as a solar treatment device.

The 1987 US EPA GS Protocol states that the specified performance and quality parameters of test waters, which vary according to the specific technology utilized by the POU/HWT system being tested, should be measured at each of the microbe challenge test points. In particular, the protocol points out the need to test the product water of units which utilize chemical disinfectants for residual concentrations of potentially toxic treatment chemicals that might exceed established maximum contaminant level guidelines. The flow rates of the devices should be monitored frequently to maintain the recommended product water production rate, as well as to detect clogging of filtration-based units and to determine the need for system cleaning or servicing. NSF Protocol P231, NSF Protocol

P248, Evaluation under the WHO Scheme and NSF/ANSI Standard 55 follow the US EPA protocol recommendations on these points.

The Chinese protocol, Guideline Relating to Drinking Water, provides little detailed information about the testing process itself. It does state that for POU devices using chemical disinfectants, the residual disinfectant concentrations should be determined at each microbe challenge point.

The NSF P248 military testing protocol calls for the monitoring of three significant parameters, pH, turbidity and temperature, three times each day during the testing process as well as at each microbe challenge point. It also points out the need to measure chemical disinfectant residuals in the product water at each microbe challenge interval.

The Evaluating Household Water Treatment Options document of WHO (2011a) advocates that testing protocols for POU/HWT devices should not only determine microbial elimination but also measure operational parameters which either influence the extent of microbe reduction or provide indications that a HWT device is operating properly. The selection of relevant key parameters to be monitored is determined primarily by the type of POU/HWT technology utilized by the device and also by expected environmental conditions that pose challenges to the microbe reduction efficiency of a system being operated in the real world. The document includes a comprehensive list and discussion of important operational parameters for each major POU/HWT technology type.

The Chinese Ministry of Health protocol mentions no non-bacteriological operational parameters to be monitored during the bacteriological testing procedure. The protocol does state that a POU/HWT device must provide product water with no more than 100 total plate count bacteria per milliliter in addition to the specified *E. coli* limit of 0 organisms/100 mL in order to be approved, but the protocol gives no indication of the source of the total plate count bacteria. The assumption is that the plate count bacteria were present in the chlorinated tap water used as the aging water and the distilled water used during the *E. coli* challenges. The Mexican protocol also mentions aerobic mesophilic bacteria as the sole non-*E. coli* test parameter to be monitored. The Mexican protocol requires that a POU device must remove 95% of the mesophilic aerobic bacteria, presumably bacteria originating in the public water supply test water. The Brazilian POU test protocol requires no operational parameters besides *E. coli* to be monitored.

17. Recommendations for Point-of-Use System Testing Protocols

Given the need for reliable POU/HWT systems which can provide improved quality drinking water for households where access to clean water is otherwise lacking, effective processes for evaluation and certification of such devices are required. A major component of the evaluation and certification process is a POU testing protocol or procedure which quantifies the microbe reduction efficiency of the device being tested under conditions which realistically predict the performance of the device in the real world. The testing protocol needs to be sufficiently detailed so as to provide a standard framework and methodology for judging not only the performance of the POU/HWT device itself, but also the validity of the testing process. At the same time, the testing protocol must possess sufficient flexibility to permit satisfactory testing of POU/HWT systems utilizing new or upgraded microbial reduction technologies and allow modifications of the protocol itself in light of newly acquired knowledge and technological capacities. The testing protocol should provide for adaptations to local public health and environmental conditions. The protocol should specify relevant, representative and readily accessible microbial types and strains and test procedures which can be conducted by laboratories possessing adequate and at least moderate levels of equipment, relevant materials and supplies and staff expertise. Such laboratories are likely to be those that can meet biosafety level 2 requirements, including for analyst proficiency.

POU/HWT testing protocols should be certified or otherwise have approval by a recognized global public health institution like the WHO or NSF-International. All proto-

cols should be well-publicized, widely disseminated and readily available, as should the materials and methods used for testing.

The following sections discuss the various components of a POU/HWT testing protocol, as outlined in Supplementary Table S1, and make recommendations for future versions of testing protocols, their methods and materials.

17.1. Addressing Microbe Reduction by POU/HWT Technologies

It would be preferable to have all existing POU/HWT microbial reduction technologies covered by a single or at least harmonized and consistent testing protocol. This comprehensive protocol should possess sufficient flexibility to allow for applicability to the wide range of available technologies and also accommodate the emergence of new or improved microbe reduction POU/HWT technologies, advances in analytical microbiology and changes in epidemiological patterns and health significance of waterborne infectious diseases. The protocol should be reviewed by an expert committee every few years for possible revision.

The revised 1987 US EPA Guide Standard and Protocol for Testing Microbiological Water Purifiers provided a well-reasoned detailed protocol that has stood the test of time to the extent that it has served as the technical foundation for subsequent testing protocols published by NSF-International, including NSF Protocol P231 Microbiological Water Purifiers (2014), NSF Protocol P248 Military Operations Microbiological Water Purifiers (2012), NSF/American National Standard Protocol 55 Ultraviolet Microbiological Water Treatment Systems (2019), as well as the WHO Harmonized Protocol V3.0 (2020). While the US EPA Guide Standard and Protocol explicitly restricted itself to ceramic filter candle, halogen-utilizing and ultraviolet lamp POU technologies, it also offered the opinion that the US EPA protocol could serve as the basis for testing HWT devices employing other types of microbial reduction technologies. The US EPA Guide Standard and Protocol also recommended that the document be reviewed for possible updating and improvement every two to three years. Hence, the US EPA Guide Standard and Protocol retains its relevance, and should continue to be used as the basis for the further development, updating and application of POU/HWT testing protocols. The fact that the 1987 GS Standard and Protocol has not been reviewed, updated or revised since its original publication is a deficiency that should now be addressed and corrected by responsible stakeholders as a priority activity.

17.2. Test Microbes

POU/HWT testing laboratories should possess the freedom to adopt either of two approaches when selecting test organisms for each of the three microbial pathogen categories, bacteria, viruses and protozoan parasites. One approach is that all test microbes should be representative and relevant enteric microorganisms of warm-blooded animals which have been shown to be transmitted by the fecal–oral route and are documented to be waterborne. The other approach is that nonpathogenic or low-risk indicator microbes could be chosen to minimize risk to laboratory personnel, reduce microbe procurement, propagation and enumeration costs, simplify the analyses, decrease time to results and avoid or minimize the need for expensive facilities, equipment and highly trained personnel. Testing laboratories which possess the necessary facilities and trained personnel could perform the POU/HWT testing with pathogenic microbes known to be of significant health concern as waterborne agents on a global basis or more specific to the region where the POU/HWT devices are likely to be deployed. Testing laboratories should be free to select a set of test microbes which could include both indicators and pathogens as well fully utilize the capabilities of the testing laboratory and its personnel.

In the case of protozoan parasites, spores of *Clostridium perfringens* or *Bacillus* spp. bacteria could serve as substitutes for protozoan cysts or oocysts when testing POU devices which utilize technologies designed to kill these resistant forms. Fluorescent plastic beads of the appropriate size and surface properties (charge and hydrophobicity) may be used

only if the POU devices being tested were designed to remove protozoan cysts and oocysts by only physical removal, such as filtration.

The US EPA Guide Standard and Protocol specified that *Klebsiella* (now *Raoultella*) *terrigena* bacteria, poliovirus 1, rotavirus and either Giardia cysts or 4 to 6 μm diameter plastic spheres be used as the test microbes. At the same time, the Guide Standard Protocol stated that the choice of test microbes should be reviewed as new microbiological and epidemiological information became available. That well-reasoned and wise recommendation seems to have anticipated and acknowledged advances in POU/HWT technologies, analytical microbiology and the evolution and increased awareness and understanding of waterborne pathogens of health concern. That review of test microbes is an unaddressed and unfulfilled need that should be addressed in the near future.

R. terrigena is a non-thermotolerant coliform living in non-polluted soils and plant habitats, it only rarely colonizes the human intestinal tract and there have been only a few cause reports of infection. The basis for its selection as a representative or surrogate for waterborne enteric bacterial pathogens remains uncertain and poorly documented [248]. Nonpathogenic strains of *E. coli* are proposed as readily accessible and easily propagated challenge bacteria by the WHO Evaluating Household Water Treatment Options document as well as the Chinese, Brazilian and Mexican test protocols, while the WHO document suggests *Campylobacter jejuni* as a well-understood reference waterborne pathogen of high public health risk. Comparison of the survival of commonly used and widely available reference strains of *E. coli* as well as recent field isolates of it in the course of disinfection or some other microbe reduction process by a POU/HWT device would inform the degree of variability exhibited by this test species. Such microbial comparisons in the context of POU/HWT performance evaluation are long overdue and still much needed.

The EPA GS Protocol specified the use of vaccine strain poliovirus type 1, as a representative human enteric virus. However, the use of live vaccine polioviruses is now discouraged and being phased out globally in light of international efforts to eradicate polioviruses from the planet. The WHO document recommends the use of a simian rotavirus as a model viral pathogen. However, the basis for choosing which rotavirus to use for testing remains unclear, as there are many different human and animal rotaviruses. The WHO document recommends an easily propagated and enumerated coliphage MS2, a bacteriophage of *E. coli*, as a nonpathogenic test virus.

In light of increased understanding of the significance of *Cryptosporidium parvum* and *Cryptosporidium hominis* as potential waterborne protozoan parasites of global public health risk and their considerable resistance to chlorine disinfection, *C. parvum*, which is widely present in cattle, is now recommended by the WHO as a more suitable choice when testing HWT devices with live protozoan parasites. However, other protozoan parasites or other highly resistant but non-pathogenic microbes as surrogates for protozoan parasites such as spores of the anaerobic bacterium *Clostridium perfringens* deserve further consideration for use in POU/HWT performance protocols.

17.3. Test Microbe Stock Production

All of the testing protocols reviewed in this document that specify preparation methods for bacterial stocks recommend culturing with incubating in nonselective broth or soft agar media overnight, which likely yields stationary phase test bacteria with a subpopulation consisting of stressed starved cells. The WHO document allows for storage and use of refrigerated stationary phase bacterial cultures for up to a week. Given that enteric bacteria excreted into surface waters are also likely starved and injured by other environmental conditions, it is possible that the use of stationary phase bacteria represent a reasonable choice, especially after days of refrigerated storage. However, further investigations of the effects of microbial and especially bacteria propagation and storage procedures on their reductions by POU/HWT systems using an agreed upon test protocol would be informative and are recommended.

The EPA Guide Standard and Protocol specifies that broth cultures of bacteria to be used for POU/HWT system testing are to be filtered through an 8 μm pore-size filter to remove aggregated bacterial cells. The WHO document also recommends elimination of bacterial aggregates by various methods. The use of non-aggregated bacterial cells is recommended for the practical purpose of achieving consistency in testing, not necessarily for reliably modeling the state of fecal bacteria cells in water. Bacterial cells in the interior of microbial aggregates may be protected to varying extents from exposure to disinfectant chemicals, and bacteria present as aggregates may be eliminated to a greater extent by filtration compared to discrete and dispersed cells. The extent of aggregation of stationary phase bacterial cultures before and after pelleting by centrifugation, washing and resuspension in buffer or test water, the typical bacterial culture preparation procedure usually performed without subsequent filtration, should be investigated and characterized on the basis of the extent to which the cells are dispersed and aggregated to varying extents. It is further recommended that the effects of these preparation procedures on the reductions of these bacteria preparations by POU/HWT processes should be determined in a representative and widely accepted testing protocol. This would determine if aggregation and/or particle association before or during the processing and preparation of bacterial cultures for use in POU/HWT testing is a common occurrence, and if filtration of the prepared stocks is necessary and effective in yielding preparations with well-defined and reproducible physical states of the cells with respect to aggregation and particle association.

Aggregation is also an issue with viruses. As with bacteria, the presence of large viral aggregates or viruses associated with or embedded in host cells or cell debris may provide protective effects against chemical disinfectants and may also influence the extent to which the viruses are removed by filtration and other physical processes dependent on particle size. However, all EPA Guide Standard-based protocols as well as the WHO Evaluation document recommend that human enteric virus stocks or coliphage virus stocks to be used for POU/HWT device testing be monodispersed by either extraction with an organic solvent or by filtration. Again, the goal of this recommendation is to achieve testing consistency rather than to realistically model the state of virus particles in water, where a certain degree of aggregation or association with host cell debris and other particulate matter would be expected. It is recommended that the effect of preparation procedures for virus stocks used for POU/HWT technology testing and the physical states of virus aggregation and particle association in these stocks should be further studied and characterized for their effects on virus reductions by different POU/HWT technologies.

Protozoan parasite cysts and oocysts (typically *Giardia* and *Cryptosporidium*, respectively) prepared for POU/HWT technology performance evaluations generally do not exhibit aggregation. This is because of the extensive multistep concentration and purification steps, including density gradient centrifugation techniques, employed for commercially produced cyst and oocyst stocks. Purchased stocks or stocks prepared using such standard techniques require little additional processing before use in POU testing, except to wash them from the storage medium and resuspend them in an appropriate test water. However, because of the evidence that waterborne protozoan cysts and oocysts may be particle-associated, further study is recommended to determine the extent to which such particle association influences their reductions by different POU/HWT technologies in standard testing protocols.

17.4. Test Microbe Quantification Methods

All of the POU/HWT test protocols reviewed in this document specify the use of selective culture media for the enumeration of challenge enteric bacteria. As discussed in earlier sections of this review, exposure of enteric bacteria to both environmental conditions outside of a host and to POU/HWT microbe reduction processes is expected to yield injured bacteria which, although still viable and potentially infectious or culturable, may not be recoverable and quantifiable following selective culture assay procedures. Consequently, the adoption of bacterial repair enumeration methods employing the appropriate choice of

culture media, plating methods and incubation conditions is highly recommended. Further research and demonstration studies are much needed and recommended to determine the most effective culture assay methods to quantify injured bacteria, focusing on the use of recommended test strains, standard testing protocols and accepted, widely used resuscitation and culture assay methods and materials.

Further investigation of the significance of light and dark repair by bacteria before, during and after POU/HWT performance evaluation utilizing UV light, solar radiation and advanced oxidation processes based on photo-Fenton-type technologies is needed. Quantification of bacteria following treatment by these types of POU/HWT systems may require the use of assay procedures allowing dark and light repair processes by injured but viable and still potentially culturable bacterial cells.

Quantification of viruses in POU/HWT technology performance evaluation testing is typically carried out by various types of infectivity assays in the appropriate type of host cells. As discussed in a previous section, quantification of viruses by molecular methods like RT-PCR and qPCR is compromised by the possibility that nucleic acids from viruses that have been rendered noninfectious by POU/HWT processes may be detected. Therefore, the use of quantitative virus infectivity assays in host cells is still recommended for POU/HWT technology performance evaluation.

Quantification of live and potentially infectious protozoan parasite cysts or oocysts by infectivity assays in either cell cultures or live animal hosts before and after exposure to POU/HWT treatment designed to kill or render them noninfectious is recommended as the basis for assay. As discussed earlier, excystation assays and staining procedures do not reliably detect only the infectious parasite cysts or oocysts, because they can also detect non-infectious ones that are no longer of human health risk concern. However, microscopic assay methods can be used effectively for quantification of fluorescent particles used as surrogates for protozoan parasite cysts and oocysts when evaluating the performance of POU/HWT technologies based on physical removal processes.

As technologies develop and more tools become available over time, more research will be needed to evaluate the ability of these advances in methods to concentrate, detect and enumerate waterborne microbes. However, an important consideration in this review of new technology is the practical application of these methods. Many molecular methods and new techniques are more technically demanding, require skilled analysts, specialized equipment and reagents, are often cost-prohibitive and are not widely available to many POU/HWT researchers and practitioners, limiting the amount of new research done as well and the incorporation of these tools and methods into standard practice. It is important to recognize the limitations of molecular methods in their inability to reliably quantify and detect infective microorganisms. Hence, culture methods remain central to the quantification of waterborne pathogens and indicator organisms for the evaluation of POU technologies.

17.5. Test Waters

To provide the bulk of the test water to quickly and efficiently operate and test a POU/HWT system throughout its recommended lifetime, a readily available source of high-quality water of near-neutral pH and low turbidity can be used. This water must be de-chlorinated or otherwise treated to eliminate all disinfecting chemicals before being used. An alternative aging water could be collected in the field at a site representative of a location where POU/HWT units might be deployed, assuming the logistical issues in obtaining sufficient volumes of the water and transporting it to the testing laboratory for use in challenge studies can be met.

However, the microbe reduction performance of POU/HWT systems must also be tested using representative worst-case, poor-quality waters. The specific composition of this worst-case water varies with the type of microbe reduction technology used by the POU/HWT device. The EPA Guide Standard and Protocol provided a well-considered detailed set of challenge waters specific to the various POU/HWT treatment technologies

addressed by that document. The WHO Recommendation document proposes the use of a standard worst-case water containing 1% untreated sewage or 20% primary wastewater effluent. Further consideration needs to be given to the composition of test waters for POU/HWT technology performance evaluation, especially on the basis of it being representative of the real-world waters to which it will be applied. This is because some of the currently specified challenge test waters are synthetic mixtures of constituents added to water that may not be representative of the typical fecally contaminated source waters encountered in many places where POU/HWT can be employed to improve drinking water quality.

17.6. Test Water Quality Parameters to Be Monitored

The composition of the test water being treated by a POU/HWT device will influence its microbe reduction efficiency. The technology utilized by a POU/HWT unit determines the relative impact and significance of each water quality parameter and the test water adjustment material, including their preparation, addition and method of analysis (for example, humic acid versus tannic acid for TOC addition). The test protocols based on the EPA Guide Standard and Protocol and the WHO Recommendation document list which water quality parameters have a significant effect on each type of POU/HWT technology. All relevant and significant parameters should be monitored and recorded for the duration of the test period. Temperature and pH affect all physical, chemical and biological processes. The turbidity level of water can play a significant role in the fate of microbes when exposed to many POU/HWT processes, which is also true for total dissolved solids and total organic carbon. For those POU/HWT technologies that employ chemicals for treatment, such as chemical disinfectants and coagulants, the concentrations of these chemicals in the test water before and after treatment must be monitored at representative intervals throughout the testing period over the total volume of water tested.

17.7. Number of Replicate POU/HWT Units Tested

The EPA Guide Standard specifies that three POU/HWT units be tested in parallel, while the WHO Recommendation document recommends two or three units. Testing three units simultaneously, with the attendant POU/HWT device aging, microbe stock production and processing, microbe quantification assays and water quality testing are challenging but manageable for an efficient work team having adequate facilities and other supporting resources. Testing devices in triplicate still provides replication of results even if one of the units malfunctions during the testing process. Therefore, POU/HWT performance evaluation testing of devices in triplicate is recommended

17.8. Test Duration

Most commercial POU/HWT systems possess a functional lifetime limit specified by the manufacturer, usually stated as the total volume of water which can be adequately treated. When a unit's lifetime has been reached, all or parts of the device need to be replaced, cleaned or otherwise serviced in order to regain or maintain its functionality. Virtually all of the POU/HWT testing protocols reviewed recognize the need to test a POU/HWT device throughout the entire operational lifetime of the unit specified by the manufacturer. This may require passing the cleaner aging water through the test units for up to 16 h per day in order to complete the lifetime volume testing process in a reasonable period of 10 to 14 days.

For those systems with indefinite and extended lifetimes like biosand filters or ceramic filters, a 14-day test period may be acceptable on a practical basis but is probably inadequate to represent an entire filter run before scouring or other cleaning. This is because biofilters may take longer to mature (ripen) and ceramic filters may operate for longer time periods before their flow rate declines to require scouring. Such filters should be operated through an entire cycle or filter run before scouring or scrubbing. Therefore, is it recommended that further evaluation of the testing period and volume of water treated be determined for

POU/HWT technologies that are designed and intended for long-term use. The test units should be allowed to rest about 8 h each day to mimic the expected daily use pattern of a POU/HWT household unit, which is probably static each night.

17.9. Microbe Challenge Schedule

The initial microbe challenge for POU/HWT devices should be immediately following the startup conditioning of the units, as recommended by the manufacturer. Periodic microbe challenges would subsequently be conducted at a reasonable number of designated fractions of the lifetime treatment volume capacity specified by the manufacturer. The EPA Guide Standard Protocol provides a challenge schedule. Initial microbe challenges can be conducted using the cleaner aging water, but the later challenges should be conducted with the worst-case poorer quality water appropriate for the type of POU/HWT treatment technology utilized. A microbe challenge following an extended stagnation period of up to 48 h during the last half of the testing period would be an appropriate challenge for POU systems using filtration, as the performance of filtration-based POU systems can be potentially degraded by intermittent flow and stagnation.

17.10. Microbe Reduction Requirements

The EPA Guide Standard and subsequent test protocols which adopted its practices established performance-based microbe reduction guidelines for each of the three classes of microbes, with requirements that bacteria, viruses and protozoan parasite cysts be reduced by six, four and three $\log_{10}$ units, respectively. These $\log_{10}$ reduction requirements were considered achievable by the POU technologies considered important and in use at the time. The intention is for the product water from a POU/HWT device to meet the microbe concentration limits of the US National Primary Drinking Water Regulations. To quantify these performance guidelines, the EPA protocol requires concentrations of challenge microbes seeded in the input test waters in excess of those which would typically be found in contaminated source waters. However, it is important to note that the microbial concentration limits and target pathogens in drinking water have evolved and expanded since the time when the 1987 GS Protocol was developed. Other regulated pathogens, such as *Legionella* spp. bacteria, human enteric viruses such as noroviruses and adenoviruses and *Cryptosporidium* spp. protozoan parasites are now recognized as important to regulate in drinking water. New US EPA regulations for the microbial quality of drinking water have since been developed, such as a series of Surface Water Treatment Rules and a Ground Water Rule.

The WHO document: Evaluating Household Water Treatment Options, provides health-risk-based microbe removal targets for POU/HWT devices. These targets are based on quantitative microbial risk assessments of three significant global waterborne pathogens considered to be reference pathogens for each microbe class: *Campylobacter jejuni* bacteria, rotaviruses and *Cryptosporidium* protozoan parasites. These QMRA analyses yielded recommended reductions for bacteria, viruses and protozoan (oo) cysts of four, five and four $\log_{10}$ units respectively, to provide highly protective water treatment, achieving an acceptable DALY health risk of 10^{-6} per person per year, based on WHO criteria. An additional lower level of performance defined as protective was also specified with lower $\log_{10}$ reduction targets and therefore acceptance of a higher DALY health risk of 10^{-4} per person per year. A third even lower level of performance called minimal protection was also specified, for which a treatment technology must meet the protective $\log_{10}$ reduction targets for two of the three classes of microbes. Provided that sufficient and accurate data are available and that the necessary assumptions are reasonable, health-risk-based microbe reduction targets provide more rational and realistic guidance to deal with real-world conditions. Furthermore, these health-risk-based microbial reductions are consistent with the WHO Guidelines for Drinking-water Quality, a consensus document widely accepted and used as the basis for health protection of drinking water in most countries throughout the world.

17.11. Other, Non-Microbial Water Quality Parameters to Be Tested and Used in Practice

In addition to the physical and chemical characteristics of the source waters listed above which need to be monitored regularly, certain quality parameters of the product water from POU/HWT systems also need to be monitored. Changes in the concentrations of these substances may provide indications of the relative microbe reduction performance of the device or indicate the level of risk to the consumer of the product water. For POU systems using disinfection to reduce microbe levels, residual disinfectant concentrations in the treated water should be routinely measured. For systems using potentially toxic metals like silver or copper to kill and inactivate microbes, these chemicals must be measured in the product water to determine if their concentrations exceed national standards or WHO Guideline values. Increasing turbidity levels in the product water of filtration-based POU devices may provide indications to the tester and the user that the microbial reduction efficiency of the device is declining or has been compromised. Regular analysis for such non-microbial water quality parameters as these provides a practical and less costly basis for regularly monitoring the quality of water treated by a POU/HWT technology.

18. Conclusions

Testing protocols for determining the ability of point-of-use HWT devices to adequately reduce the concentrations of waterborne microorganisms of public health significance must be written in such a way as to provide a robust, detailed and reproducible regime that tests these devices under conditions that model real-world situations where POU/HWT units are likely to be utilized, while also offering some portability of certain technologies and generalizability of the data. The protocols need to allow flexibility to deal with different water treatment technologies, a variety of test microbe choices, variable capabilities of testing facilities and differing regional public health situations, water sources and water quality contexts and conditions.

However, the testing protocols also need to be written in sufficient detail to accurately and reproducibly evaluate the performance of POU/HWT devices, thereby maintaining the confidence of public health professionals, regulators, POU/HWT manufacturers and consumers of the quality of the product water provided by these systems. Protocols must require the testing of replicate units in parallel through the entire (or as long as practical) functional lifetime of the units being tested using both clean aging water and realistically poor-quality water by challenging periodically with pathogenic microbes, indicator microbes or microbe surrogates from all three classes of microorganisms. The quantification of bacteria should be conducted using techniques which enhance the recovery of injured cells, and the enumeration of viruses and protozoan parasites should be performed by infectivity assays if possible, especially for treatment processes based on chemical and physical disinfection. In addition to the quantification of microbe reduction, other significant water quality parameters which influence the relative microbe reduction efficiency by the various types of water treatment technologies and provide indications of the performance of the devices or affect the quality of the product water (residual disinfectants or heavy metals) must be monitored as well.

The EPA Guide Standard and Protocol, then later NSF protocols that adopted the EPA GS testing format, the WHO Evaluating Household Water Treatment Options document and the protocol of the WHO Scheme to Evaluate HWT Technologies all describe and discuss the features of appropriate laboratory practices in testing protocols for POU/HWT devices in great detail and satisfy these testing requirements to varying degrees. Some are very specific protocols with detailed preparation and testing methods. Since the initial development and promulgation of the EPA GS Protocol in 1987, nearly all of the subsequent standards, guidelines and recommendations for performance evaluation by NSF-International and WHO have been based on these documents. There is a continuum of adaptations and improvements in these documents that have made them widely used worldwide. Unfortunately, some country-specific documents lack the breadth, completeness and rigor of protocols, procedures, methods and materials that are consistent with the

standards and guidance of these authoritative sources. Countries seeking to develop their own standards, protocols and certification programs are encouraged to model and adapt them according to those of WHO, NSF-International and US EPA.

However, all of the existing protocols should be further improved and refined by development and adoption of standardized microbial stock propagation and preparation methods which would yield microbes in a consistent state of aggregation and physiological stress to better model the conditions of waterborne enteric microbes in the real world. Quantification methods for bacteria exhibiting physiological injury also need to be further improved and adopted as standard components of POU/HWT testing protocols. The critical role of safe handling and safe storage of product water from POU/HWT devices must be understood and acknowledged by POU/HWT system designers and manufacturers, distributors of these systems and those who rely on these devices to provide safer water for themselves and their families. Finally, the need for harmonized, performance-based guidelines and national standards with appropriate POU/HWT testing protocols that are implementable in laboratories in countries around the world is critical to protect consumers from inferior and inadequate technologies that do not provide consumers with safe household water and put them at risk of waterborne disease. There is a need for all POU/HWT stakeholders to work together and further improve and harmonize what now exists for technology performance evaluation and make such testing more widely available and achievable worldwide.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13081094/s1, Table S1: Point-of-Use Testing Protocols and their Methods Reviewed, Table S2.

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References

1. *Global WASH Fast Facts*; Centers for Disease Control and Prevention: Atlanta, GA, USA, 2018.
2. UNICEF. *One Is Too Many: Ending Child Deaths from Pneumonia and Diarrhoea*; UNICEF: New York, NY, USA, 2016; pp. 1–74.
3. Fewtrell, L.; Kaufmann, R.B.; Kay, D.; Enanoria, W.; Haller, L.; Colford, J.M. Water, sanitation, and hygiene interventions to reduce diarrhoea in less developed countries: A systematic review and meta-analysis. *Lancet Infect. Dis.* **2005**, *5*, 42–52. [CrossRef]
4. Clasen, T.; Schmidt, W.-P.; Rabie, T.; Roberts, I.; Cairncross, S. Interventions to improve water quality for preventing diarrhoea: Systematic review and meta-analysis. *BMJ* **2007**, *334*, 782. [CrossRef] [PubMed]
5. Sobsey, M.D.; World Health Organization. *Managing Water in the Home: Accelerated Health Gains from Improved Water Supply*; World Health Organization: Geneva, Switzerland, 2002.
6. Obsey, M.D.S.; Tauber, C.E.S.; Asanova, L.M.C.; Brown, J.M.; Elliott, M.A. Point of Use Household Drinking Water Filtration: A Practical, Effective Solution for Providing Sustained Access to Safe Drinking Water in the Developing World. *Environ. Sci. Technol.* **2008**, *42*, 4261–4267. [CrossRef]
7. World Health Organization. *Evaluating Household Water Treatment Options: Health-Based Targets and Microbiological Performance Specifications*; World Health Organization: Geneva, Switzerland, 2011; 59p.
8. Pooi, C.K.; Ng, H.Y. Review of low-cost point-of-use water treatment systems for developing communities. *NPJ Clean Water* **2018**, *1*, 11. [CrossRef]
9. WHO. *Status of National Household Water Treatment and Safe Storage Policies in Selected Countries*; World Health Organization: Geneva, Switzerland, 2012.
10. Ministry of Health of the People's Republic of China. *Microbial Standard for Drinking Water Purifier*; GB/T 5750-2006; Ministry of Health of the People's Republic of China: Beijing, China, 2010.

11. Ministry of Health of the People's Republic of China. Guideline for Declaration of Health Administration Permission of Products Relating to Health and Safety of Drinking Water. Available online: https://www.who.int/water_sanitation_health/dwq/gdwq0506.pdf (accessed on 15 January 2021).
12. WHO. *WHO International Scheme to Evaluate Household Water Treatment Technologies*; World Health Organization: Geneva, Switzerland, 2014.
13. WHO. *Results of Round I of the WHO International Scheme to Evaluate Household Water Treatment Technologies*; World Health Organization: Geneva, Switzerland, 2016.
14. NSF International. *NSF Protocol P248.01: Military Operations Microbiological Water Purifiers*; NSF International: Ann Arbor, MI, USA, 2012.
15. NSF International. *NSF Protocol P231: Microbiological Water Purifiers*; NSF International: Ann Arbor, MI, USA, 2014.
16. NSF International. *NSF Protocol 62-2019: Drinking Water Distillation Systems*; NSF International: Ann Arbor, MI, USA, 2019.
17. NSF International. *NSF Protocol 55-2019: Ultraviolet Microbiological Water Treatment Systems*; NSF International: Ann Arbor, MI, USA, 2019.
18. NSF International. *NSF Standard 244-2019: Supplemental Municipal Water Treatment Systems-Filtration*; NSF International: Ann Arbor, MI, USA, 2019.
19. NSF International. *NSF Protocol 53-2019: Drinking Water Treatment Units-Health Effects*; NSF International: Ann Arbor, MI, USA, 2019.
20. NSF International. *NSF Protocol 58-2019: Reverse Osmosis Drinking Water Treatment Systems*; NSF International: Ann Arbor, MI, USA, 2019.
21. Water Quality India Association. *Certification Scheme for Drinking Water Treatment Systems That Make Microbiological Reduction Claims*; Water Quality India Association: Mumbai, India, 2015.
22. USEPA. *Guide Standard and Protocol for Testing Microbiological Water Purifiers*; Report of Task Force; USEPA: Washington, DC, USA, 1987.
23. World Health Organization. *Guidelines for Drinking-Water Quality*; World Health Organization: Geneva, Switzerland, 2011; 541p.
24. American Public Health Association. *Standard Methods for the Examination of Water and Wastewater*, 22nd ed.; American Public Health Association: Washington, DC, USA, 2012; 724p.
25. Dai, X.; Hozalski, R.M. Evaluation of Microspheres as Surrogates forCryptosporidium parvumOocysts in Filtration Experiments. *Environ. Sci. Technol.* **2003**, *37*, 1037–1042. [CrossRef]
26. Sobsey, M.D.; Battigelli, D.A.; Shin, G.A.; Newland, S. RT-PCR amplification detects inactivated viruses in water and wastewater. *Water Sci. Technol.* **1998**, *38*, 91–94. [CrossRef]
27. Josephson, K.L.; Gerba, C.P.; Pepper, I.L. Polymerase chain reaction detection of nonviable bacterial pathogens. *Appl. Environ. Microbiol.* **1993**, *59*, 3513–3515. [CrossRef]
28. Wagner-Wiening, C.; Kimmig, P. Detection of viable Cryptosporidium parvum oocysts by PCR. *Appl. Environ. Microbiol.* **1995**, *61*, 4514–4516. [CrossRef]
29. Schwab, K.J.; De Leon, R.; Sobsey, M.D. Immunoaffinity concentration and purification of waterborne enteric viruses for detection by reverse transcriptase PCR. *Appl. Environ. Microbiol.* **1996**, *62*, 2086–2094. [CrossRef]
30. Fittipaldi, M.; Rodriguez, N.J.P.; Codony, F.; Adrados, B.; Peñuela, G.A.; Morató, J. Discrimination of infectious bacteriophage T4 virus by propidium monoazide real-time PCR. *J. Virol. Methods* **2010**, *168*, 228–232. [CrossRef]
31. Pecson, B.M.; Martin, L.V.; Kohn, T. Quantitative PCR for Determining the Infectivity of Bacteriophage MS2 upon Inactivation by Heat, UV-B Radiation, and Singlet Oxygen: Advantages and Limitations of an Enzymatic Treatment to Reduce False-Positive Results. *Appl. Environ. Microbiol.* **2009**, *75*, 5544–5554. [CrossRef]
32. Nuanualsuwan, S.; Cliver, D.O. Pretreatment to avoid positive RT-PCR results with inactivated viruses. *J. Virol. Methods* **2002**, *104*, 217–225. [CrossRef]
33. Campbell, A.T.; Robertson, L.J.; Smith, H.V. Viability of Cryptosporidium parvum oocysts: Correlation of in vitro excystation with inclusion or exclusion of fluorogenic vital dyes. *Appl. Environ. Microbiol.* **1992**, *58*, 3488–3493. [CrossRef]
34. Black, E.K.; Taghi-Kilani, R.; Finch, G.R.; Belosevic, M. Comparison of assays for Cryptosporidium parvum oocysts viability after chemical disinfection. *FEMS Microbiol. Lett.* **1996**, *135*, 187–189. [CrossRef]
35. Shin, G.-A.; Linden, K.G.; Arrowood, M.J.; Sobsey, M.D. Low-Pressure UV Inactivation and DNA Repair Potential of Cryptosporidium parvum Oocysts. *Appl. Environ. Microbiol.* **2001**, *67*, 3029–3032. [CrossRef]
36. Liang, Z.; Keeley, A. Comparison of propidium monoazide-quantitative PCR and reverse transcription quantitative PCR for viability detection of fresh Cryptosporidium oocysts following disinfection and after long-term storage in water samples. *Water Res.* **2012**, *46*, 5941–5953. [CrossRef]
37. National Research Council. *Indicators for Waterborne Pathogens*; National Academies Press: Washington, DC, USA, 2004; 328p. [CrossRef]
38. National Research Council. *Setting Priorities for Drinking Water Contaminants*; National Academies Press: Washington, DC, USA, 1999; 128p.
39. Blackburn, B.G.; Craun, G.F.; Yoder, J.S.; Hill, V.; Calderon, R.L.; Chen, N.; Lee, S.H.; Levy, D.A.; Beach, M.J. Surveillance for waterborne-disease outbreaks associated with drinking water–United States, 2001-2002. *MMWR. Surveill. Summ.* **2004**, *53*, 23–45.

40. Craun, G.F.; McCabe, L.J. Review of the Causes of Waterborne-Disease Outbreaks. *J. Am. Water Works Assoc.* **1973**, *65*, 74–84. [CrossRef]
41. World Health Organization. *Surveillance and Outbreak Management of Water-Related Infectious Diseases Associated with Water-Supply Systems*; Licence: CC BY-NC-SA 3.0 IGO; WHO Regional Office for Europe: Copenhagen, Denmark, 2019.
42. Smith, T. *A New Method for Determining Quantitatively the Pollution of Water by Fecal Bacteria*; Thirteenth Annual Report of the State Board of Health of New York for 712; New York State Department of Health: New York, NY, USA, 1892; p. 1893.
43. Burlage, R.S. *Principles of Public Health Microbiology*; Jones & Bartlett Publishers: Burlington, MA, USA, 2012; 417p.
44. Ashbolt, N.J.; Grabow, W.O.K.; Snozzi, M. Indicators of microbial water quality. In *Water Quality: Guidelines, Standards and Health*; World Health Organization: Geneva, Switzerland, 2001; pp. 289–316.
45. WHO. *National Workshop on Evaluating Household Water Treatment Performance and Scaling up Safe-Drinking Water Solutions*; World Health Organization: Geneva, Switzerland, 2013.
46. World Health Organization. Health Organization. Health criteria and other supporting information. In *Guidelines for Drinking-Water Quality*, 2nd ed.; World Health Organization: Geneva, Switzerland, 1996; Volume 2.
47. World Health Organization. *Water Quality: Guidelines, Standards, and Health: Assessment of Risk and Risk Management for Water-related Infectious Disease*; World Health Organization: Geneva, Switzerland, 2001; 424p.
48. Gertjan, M.; Payment, P.; Alfred, D.; Robertson, W.; Waite, M.; Paul, H.; Roy, K.; Andersson, Y. Safe drinking water: An ongoing challenge. In *Assessing Microbial Safety of Drinking Water*; World Health Organization: Geneva, Switzerland, 2003; p. 11.
49. Geldreich, E.E.; Huff, C.B.; Bordner, R.H.; Kabler, P.W.; Clark, H.F. The faecal coli-aerogenes flora of soils from various geographical areas. *J. Appl. Bacteriol.* **1962**, *25*, 87–93. [CrossRef]
50. Dufour, A.P.; Cabelli, V.J. Membrane Filter Procedure for Enumerating the Component Genera of the Coliform Group in Seawater. *Appl. Microbiol.* **1975**, *29*, 826–833. [CrossRef]
51. Dufour, A.P. Escherichia coli: The Fecal Coliform. In *Bacterial Indicators/Health Hazards Associated with Water*; Hoadley, A., Dutka, B., Eds.; ASTM International: West Conshohocken, PA, USA, 1977; pp. 48–58. [CrossRef]
52. Report of the medical officer of the local government board for 1899–1900. *Lancet* **1901**, *157*, 1779–1780. [CrossRef]
53. Slanetz, L.W.; Bartley, C.H. Numbers of Enterococci in Water, Sewage, and Feces Determined by the Membrane Filter Technique with an Improved Medium1. *J. Bacteriol.* **1957**, *74*, 591–595. [CrossRef]
54. Bordalo, A.; Onrassami, R.; Dechsakulwatana, C. Survival of faecal indicator bacteria in tropical estuarine waters (Bangpakong River, Thailand). *J. Appl. Microbiol.* **2002**, *93*, 864–871. [CrossRef] [PubMed]
55. Risebro, H.L.; Breton, L.; Aird, H.; Hooper, A.; Hunter, P.R. Contaminated Small Drinking Water Supplies and Risk of Infectious Intestinal Disease: A Prospective Cohort Study. *PLoS ONE* **2012**, *7*, e42762. [CrossRef] [PubMed]
56. American Water Works. *Waterborne Pathogens*; American Water Works: Camden, NJ, USA, 1999; 285p.
57. Gall, A.M.; Mariñas, B.J.; Lu, Y.; Shisler, J.L. Waterborne Viruses: A Barrier to Safe Drinking Water. *PLOS Pathog.* **2015**, *11*, e1004867. [CrossRef] [PubMed]
58. Kott, Y. Estimation of low numbers of *Escherichia coli* bacteriophage by use of the most probable number method. *Appl. Microbiol.* **1966**, *14*, 141–144. [CrossRef] [PubMed]
59. Grabow, W. Bacteriophages: Update on application as models for viruses in water. *Water SA* **2004**, *27*, 251–268. [CrossRef]
60. Havelaar, A.H.; Van Olphen, M.; Drost, Y.C. F-specific RNA bacteriophages are adequate model organisms for enteric viruses in fresh water. *Appl. Environ. Microbiol.* **1993**, *59*, 2956–2962. [CrossRef]
61. Skraber, S.; Gassilloud, B.; Gantzer, C. Comparison of Coliforms and Coliphages as Tools for Assessment of Viral Contamination in River Water. *Appl. Environ. Microbiol.* **2004**, *70*, 3644–3649. [CrossRef]
62. USEPA. *Method 1602: Male-Specific (F+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure*; EPA 821-R-01-029; USEPA: Washington, DC, USA, 2001.
63. Lamy, M.C.; Sanseverino, I.; Niegowska, M.; Lettieri, T. *Microbiological Parameters under the Drinking Water Directive*; Publications Office of the European Union: Luxembourg, 2020.
64. Sinclair, R.G.; Rose, J.B.; Hashsham, S.A.; Gerba, C.P.; Haas, C.N. Criteria for Selection of Surrogates Used to Study the Fate and Control of Pathogens in the Environment. *Appl. Environ. Microbiol.* **2012**, *78*, 1969–1977. [CrossRef]
65. Marshall, M.M.; Naumovitz, D.; Ortega, Y.; Sterling, C.R. Waterborne protozoan pathogens. *Clin. Microbiol. Rev.* **1997**, *10*, 69–70. [CrossRef]
66. Barrell, R.A.; Hunter, P.R.; Nichols, G. Microbiological standards for water and their relationship to health risk. *Commun. Dis. Public Health* **2000**, *3*, 8–13.
67. Rice, E.W.; Fox, K.R.; Miltner, R.J.; Lytle, D.A.; Johnson, C.H. Evaluating plant performance with endospores. *J. Am. Water Work. Assoc.* **1996**, *88*, 122–130. [CrossRef]
68. Brown, J.; Chai, R.; Wang, A.; Sobsey, M.D. Microbiological Effectiveness of Mineral Pot Filters in Cambodia. *Environ. Sci. Technol.* **2012**, *46*, 12055–12061. [CrossRef]
69. Garvey, M.; Clifford, E.; O'Reilly, E.; Rowan, N.J. Efficacy of Using HarmlessBacillusEndospores to Estimate the Inactivation ofCryptosporidium parvumOocysts in Water. *J. Parasitol.* **2013**, *99*, 448–452. [CrossRef]
70. Mamane-Gravetz, H.; Linden, K.G.; Cabaj, A.; Sommer, R. Spectral sensitivity of Bacillus subtilis spores and MS2 coliphage for validation testing of ultraviolet reactors for water disinfection. *Environ. Sci. Technol.* **2005**, *39*, 7845–7852. [CrossRef]

71. Allen, J.M.; Clancy, J.L.; Rice, E.W. Pathogen monitoring—old baggage from the last millennium. *J. Am. Water Works Assoc.* **2000**, *92*, 64–76. [CrossRef]
72. Sproul, O.J. Removal of viruses by treatment processes. In *Viruses in Water*; American Public Health Association: Washington, DC, USA, 1976; pp. 167–179.
73. LeChevallier, M.W.; Au, K.K.; World Health Organization. *Water Treatment and Pathogen Control: Process Efficiency in Achieving Safe Drinking-Water*; World Health Organization: Geneva, Switzerland, 2004; 112p.
74. Hijnen, W.A.M.; Beerendonk, E.F.; Medema, G.J. Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: A review. *Water Res.* **2006**, *40*, 3–22. [CrossRef]
75. Sobsey, M.D. Inactivation of Health-Related Microorganisms in Water by Disinfection Processes. *Water Sci. Technol.* **1989**, *21*, 179–195. [CrossRef]
76. Chang, J.C.; Ossoff, S.F.; Lobe, D.C.; Dorfman, M.H.; Dumais, C.M.; Qualls, R.G.; Johnson, J.D. UV inactivation of pathogenic and indicator microorganisms. *Appl. Environ. Microbiol.* **1985**, *49*, 1361–1365. [CrossRef]
77. Gerba, P.C.; Nwachuku, N.; Riley, K.R. Disinfection resistance of waterborne pathogens on the United States Environmental Protection Agency's Contaminant Candidate List (CCL). *J. Water Supply Res. Technol. Aqua* **2003**, *52*, 81–94. [CrossRef]
78. Morris, J.C. Aspects of the Quantitative Assessment of Germicidal Effciency. In *Disinfection: Water and Wastewater*; Ann Arbor Science Publishers: Canberra, Australia, 1975.
79. Betancourt, W.Q.; Rose, J.B. Drinking water treatment processes for removal of Cryptosporidium and Giardia. *Vet. Parasitol.* **2004**, *126*, 219–234. [CrossRef]
80. Michen, B.; Meder, F.; Rust, A.; Fritsch, J.; Aneziris, C.; Graule, T. Virus Removal in Ceramic Depth Filters Based on Diatomaceous Earth. *Environ. Sci. Technol.* **2011**, *46*, 1170–1177. [CrossRef]
81. Hijnen, W.; Schijven, J.; Bonné, P.; Visser, A.; Medema, G. Elimination of viruses, bacteria and protozoan oocysts by slow sand filtration. *Water Sci. Technol.* **2004**, *50*, 147–154. [CrossRef]
82. Liu, O.C.; Seraichekas, H.R.; Akin, E.W.; Brashear, D.A.; Katz, E.L. Relative resistance of twenty human enteric viruses to free chlorine in Potomac water. In Proceedings of the 13th Water Quality Conference, University of Illinois, Urbana, IL, USA, 13–17 February 1971.
83. Engelbrecht, R.S.; Weber, M.J.; Salter, B.L.; Schmidt, C.A. Comparative inactivation of viruses by chlorine. *Appl. Environ. Microbiol.* **1980**, *40*, 249–256. [CrossRef]
84. Shin, G.-A.; Linden, K.G.; Sobsey, M.D. Low pressure ultraviolet inactivation of pathogenic enteric viruses and bacteriophages. *J. Environ. Eng. Sci.* **2005**, *4*, S7–S11. [CrossRef]
85. Liu, O.C.; McGowan, F. *Effect of Chlorination on Human Enteric Viruses in Partially Treated Water from the Potomac River Estuary*; Environmental Protection Agency: Washington, DC, USA, 1971.
86. Payment, P.; Tremblay, M.; Trudel, M. Relative resistance to chlorine of poliovirus and coxsackievirus isolates from environmental sources and drinking water. *Appl. Environ. Microbiol.* **1985**, *49*, 981–983. [CrossRef]
87. Harakeh, M.; Butler, M. Inactivation of human rotavirus, SA11 and other enteric viruses in effluent by disinfectants. *J. Hyg.* **1984**, *93*, 157–163. [CrossRef]
88. Finch, G.R.; Fairbairn, N. Comparative inactivation of poliovirus type 3 and MS2 coliphage in demand-free phosphate buffer by using ozone. *Appl. Environ. Microbiol.* **1991**, *57*, 3121–3126. [CrossRef]
89. Gerba, C.P.; Gramos, D.M.; Nwachuku, N. Comparative Inactivation of Enteroviruses and Adenovirus 2 by UV Light. *Appl. Environ. Microbiol.* **2002**, *68*, 5167–5169. [CrossRef]
90. Nwachuku, N.; Gerba, C.P.; Oswald, A.; Mashadi, F.D. Comparative Inactivation of Adenovirus Serotypes by UV Light Disinfection. *Appl. Environ. Microbiol.* **2005**, *71*, 5633–5636. [CrossRef] [PubMed]
91. Zerda, K.S.; Gerba, C.P.; Hou, K.C.; Goyal, S.M. Adsorption of viruses to charge-modified silica. *Appl. Environ. Microbiol.* **1985**, *49*, 91–95. [CrossRef] [PubMed]
92. Gerba, C.; Wallis, C.; Melnick, J. Viruses in Water: The problem, some solutions. *Environ. Sci. Technol.* **1975**, *9*, 1122–1126. [CrossRef]
93. Sproul, O.J. *Critical Review of Virus Removal by Coagulation Processes and pH Modifications*; United States Environmental Protection Agency: Washington, DC, USA, 1980.
94. Payment, P.; Armon, R.; Gerba, C.P. Virus removal by drinking water treatment processes. *Crit. Rev. Environ. Control.* **1989**, *19*, 15–31. [CrossRef]
95. Abbaszadegan, M.; Mayer, B.K.; Ryu, H.; Nwachuku, N. Efficacy of Removal of CCL Viruses under Enhanced Coagulation Conditions. *Environ. Sci. Technol.* **2007**, *41*, 971–977. [CrossRef]
96. Shirasaki, N.; Matsushita, T.; Matsui, Y.; Urasaki, T.; Ohno, K. Comparison of behaviors of two surrogates for pathogenic waterborne viruses, bacteriophages Qβ and MS2, during the aluminum coagulation process. *Water Res.* **2009**, *43*, 605–612. [CrossRef]
97. Hayes, S.; Sivaganesan, M.; White, K.; Pfaller, S. Assessing the effectiveness of low-pressure ultraviolet light for inactivatingMycobacterium aviumcomplex (MAC) micro-organisms. *Lett. Appl. Microbiol.* **2008**, *47*, 386–392. [CrossRef]
98. Shin, G.-A.; Lee, J.-K.; Freeman, R.; Cangelosi, G.A. Inactivation of Mycobacterium avium Complex by UV Irradiation. *Appl. Environ. Microbiol.* **2008**, *74*, 7067–7069. [CrossRef]

99. EPA. National Primary Drinking Water Regulations: Long Term 1 Enhanced Surface Water Treatment Rule. Final Rule. *Fed. Regist.* **2002**, *67*, 1811–1844.
100. Taylor, R.H.; Falkinham, J.O.; Norton, C.D.; Lechevallier, M.W. Chlorine, Chloramine, Chlorine Dioxide, and Ozone Susceptibility of Mycobacterium avium. *Appl. Environ. Microbiol.* **2000**, *66*, 1702–1705. [CrossRef]
101. Blaser, M.J.; Smith, P.F.; Wang, W.L.; Hoff, J.C. Inactivation of Campylobacter jejuni by chlorine and monochloramine. *Appl. Environ. Microbiol.* **1986**, *51*, 307–311. [CrossRef]
102. Sinton, L.; Hall, C.; Braithwaite, R. Sunlight inactivation of Campylobacter jejuni and Salmonella enterica, compared with Escherichia coli, in seawater and river water. *J. Water Health* **2007**, *5*, 357–365. [CrossRef]
103. Boyle, M.; Sichel, C.; Fernández-Ibáñez, P.; Arias-Quiroz, G.B.; Iriarte-Puña, M.; Mercado, A.; Ubomba-Jaswa, E.; McGuigan, K.G. Bactericidal Effect of Solar Water Disinfection under Real Sunlight Conditions. *Appl. Environ. Microbiol.* **2008**, *74*, 2997–3001. [CrossRef]
104. Massa, S.; Armuzzi, R.; Tosques, M.; Canganella, F.; Trovatelli, L.D. Susceptibility to chlorine of Aeromonas hydrophila strains. *J. Appl. Microbiol.* **1999**, *86*, 169–173. [CrossRef]
105. Hijnen, W.; Van Der Veer, A.; Beerendonk, E.; Medema, G. Increased resistance of environmental anaerobic spores to inactivation by UV. *Water Supply* **2004**, *4*, 55–61. [CrossRef]
106. Heaselgrave, W.; Kilvington, S. The efficacy of simulated solar disinfection (SODIS) against Ascaris, Giardia, Acanthamoeba, Naegleria, Entamoeba and Cryptosporidium. *Acta Trop.* **2011**, *119*, 138–143. [CrossRef]
107. McGuigan, K.; Mendez-Hermida, F.; Castro-Hermida, J.; Ares-Mazas, E.; Kehoe, S.; Boyle, M.; Sichel, C.; Fernandez-Ibanez, P.; Meyer, B.; Ramalingham, S.; et al. Batch solar disinfection inactivates oocysts of Cryptosporidium parvum and cysts of Giardia muris in drinking water. *J. Appl. Microbiol.* **2006**, *101*, 453–463. [CrossRef]
108. Dai, X.; Boll, J. Evaluation of Attachment of Cryptosporidium parvum and Giardia lamblia to Soil Particles. *J. Environ. Qual.* **2003**, *32*, 296–304. [CrossRef]
109. Jacangelo, J.G.; Adham, S.S.; Laîné, J.-M. Mechanism of Cryptosporidium, Giardia, and MS2 virus removal by MF and UF. *J. Am. Water Work. Assoc.* **1995**, *87*, 107–121. [CrossRef]
110. Chauret, C.P.; Radziminski, C.Z.; LePuil, M.; Creason, R.; Andrews, R.C. Chlorine Dioxide Inactivation ofCryptosporidium parvum Oocysts and Bacterial Spore Indicators. *Appl. Environ. Microbiol.* **2001**, *67*, 2993–3001. [CrossRef]
111. Shaffer, P.T.; Metcalf, T.G.; Sproul, O.J. Chlorine resistance of poliovirus isolants recovered from drinking water. *Appl. Environ. Microbiol.* **1980**, *40*, 1115–1121. [CrossRef]
112. Lee, H.S.; Sobsey, M.D. Survival of prototype strains of somatic coliphage families in environmental waters and when exposed to UV low-pressure monochromatic radiation or heat. *Water Res.* **2011**, *45*, 3723–3734. [CrossRef]
113. WHO. *Round II of the Scheme*; WHO: Geneva, Switzerland, 2019.
114. Harris, G.D.; Adams, V.; Sorensen, D.L.; Curtis, M.S. Ultraviolet inactivation of selected bacteria and viruses with photoreactivation of the bacteria. *Water Res.* **1987**, *21*, 687–692. [CrossRef]
115. Clancy, J.L.; Bukhari, Z.; Hargy, T.M.; Bolton, J.R.; Dussert, B.W.; Marshall, M.M. Using UV to inactivate Cryptosporidium. *J. Am. Water Works Assoc.* **2000**, *92*, 97–104. [CrossRef]
116. Craik, S. Inactivation of Giardia muris cysts using medium-pressure ultraviolet radiation in filtered drinking water. *Water Res.* **2000**, *34*, 4325–4332. [CrossRef]
117. LeChevallier, M.W.; Au, K.-K. *Water Treatment and Pathogen Control*; IWA Publishing: London, UK, 2004; 136p.
118. Smeets, P.; Rietveld, L.; Hijnen, W.; Medema, G.; Stenström, T. Efficacy of Water Treatment Processes. In *Microrisk Report*; European Commission: Brussels, Belgium, 2006.
119. Thompson, M.; Ellison, S.L.R.; Wood, R. *The Harmonized Protocol*; Organization of Proficiency Testing Schemes, IUPAC: Research Triangle Park, NC, USA, 2016.
120. Mattern, C.F.; Daniel, W.A. Replication of poliovirus in HeLa cells: Electron microscopic observations. *Virology* **1965**, *26*, 646–663. [CrossRef]
121. Mukoyama, A.; Hagiwara, A.; Tsuruhara, T. Intracytoplasmic Crystals of Enterovirus 71 in Cultured Cells. *J. Electron Microsc.* **1981**, *30*, 67–68. [CrossRef]
122. Williams, F.P. Membrane-associated viral complexes observed in stools and cell culture. *Appl. Environ. Microbiol.* **1985**, *50*, 523–526. [CrossRef]
123. Saif, L.J.; Theil, K.W.; Bohl, E.H. Morphogenesis of Porcine Rotavirus in Porcine Kidney Cell Cultures and Intestinal Epithelial Cells. *J. Gen. Virol.* **1978**, *39*, 205–217. [CrossRef]
124. Young, D.C.; Sharp, D.G. Poliovirus aggregates and their survival in water. *Appl. Environ. Microbiol.* **1977**, *33*, 168–177. [CrossRef]
125. Mattle, M.J.; Crouzy, B.; Brennecke, M.; Wigginton, K.R.; Perona, P.; Kohn, T. Impact of Virus Aggregation on Inactivation by Peracetic Acid and Implications for Other Disinfectants. *Environ. Sci. Technol.* **2011**, *45*, 7710–7717. [CrossRef]
126. Floyd, R.; Johnson, J.D.; Sharp, D.G. Inactivation by bromine of single poliovirus particles in water. *Appl. Environ. Microbiol.* **1976**, *31*, 298–303. [CrossRef]
127. Schnaitman, C.A.; Klena, J.D. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiol. Rev.* **1993**, *57*, 655–682. [CrossRef]
128. Reilly, J.K.; Kippin, J.S. Relationship of bacterial counts with turbidity and free chlorine in two distribution systems. *J. Am. Water Works Assoc.* **1983**, *75*, 309–312. [CrossRef]

129. Thurlow, L.R.; Thomas, V.C.; Hancock, L.E. Capsular Polysaccharide Production in Enterococcus faecalis and Contribution of CpsF to Capsule Serospecificity. *J. Bacteriol.* **2009**, *191*, 6203–6210. [CrossRef]
130. Nikaido, H. Structure and Functions of the Cell Envelope of Gram-Negative Bacteria. *Clin. Infect. Dis.* **1988**, *10*, S279–S281. [CrossRef]
131. Dzul, S.P.; Thornton, M.M.; Hohne, D.N.; Stewart, E.J.; Shah, A.A.; Bortz, D.M.; Solomon, M.J.; Younger, J.G. Contribution of theKlebsiella pneumoniaeCapsule to Bacterial Aggregate and Biofilm Microstructures. *Appl. Environ. Microbiol.* **2011**, *77*, 1777–1782. [CrossRef]
132. Olson, B.H.; Stewart, M. Factors that change bacterial resistance to disinfection. In *Water Chlorination: Chemistry, Environmental Impact and Health Effects*; Lewis Publishers: New York, NY, USA, 1987; Volume 6, pp. 885–904.
133. Stewart, M.H.; Olson, B.H. Impact of growth conditions on resistance of Klebsiella pneumoniae to chloramines. *Appl. Environ. Microbiol.* **1992**, *58*, 2649–2653. [CrossRef]
134. Carlson, S.; Hässelbarth, U.; Langer, R. Water disinfection by means of chlorine: Killing of aggregate bacteria (author's transl). *Zent. Bakteriol. Orig. B* **1975**, *161*, 233–247.
135. Berman, D.; Rice, E.W.; Hoff, J.C. Inactivation of particle-associated coliforms by chlorine and monochloramine. *Appl. Environ. Microbiol.* **1988**, *54*, 507–512. [CrossRef]
136. Behnke, S.; Parker, A.E.; Woodall, D.; Camper, A.K. Comparing the Chlorine Disinfection of Detached Biofilm Clusters with Those of Sessile Biofilms and Planktonic Cells in Single- and Dual-Species Cultures. *Appl. Environ. Microbiol.* **2011**, *77*, 7176–7184. [CrossRef]
137. Lechevallier, M.W.; Cawthon, C.D.; Lee, R.G. Factors promoting survival of bacteria in chlorinated water supplies. *Appl. Environ. Microbiol.* **1988**, *54*, 649–654. [CrossRef]
138. Bichai, F.; Léveillé, S.; Barbeau, B. Comparison of the role of attachment, aggregation and internalisation of microorganisms in UVC and UVA (solar) disinfection. *Water Sci. Technol.* **2011**, *63*, 1823–1831. [CrossRef]
139. Bohrerova, Z.; Linden, K.G. Ultraviolet and Chlorine Disinfection of Mycobacterium in Wastewater: Effect of Aggregation. *Water Environ. Res.* **2006**, *78*, 565–571. [CrossRef]
140. Berg, J.D.; Matin, A.; Roberts, P.V. Effect of antecedent growth conditions on sensitivity of Escherichia coli to chlorine dioxide. *Appl. Environ. Microbiol.* **1982**, *44*, 814–819. [CrossRef]
141. Wei, H.J.; Chang, S.L. A multi-Poisson distribution model for treating disinfection data. In *Disinfection: Water and Wastewater*; Ann Arbor Science Publishers: Canberra, Australia, 1975; p. 11.
142. Butkus, M.A.; Bays, J.T.; Labare, M.P. Influence of Surface Characteristics on the Stability of Cryptosporidium parvum Oocysts. *Appl. Environ. Microbiol.* **2003**, *69*, 3819–3825. [CrossRef]
143. Iyer, V.S.; Majumdar, U.; Waskar, M.; Dagaonkar, M.V. Aggregation kinetics of Cryptosporidium parvum oocysts. *J. Environ. Chem. Eng.* **2013**, *1*, 504–509. [CrossRef]
144. Berg, G. Removal of viruses from sewage, effluents, and waters. I. A review. *Bull. World Health Organ.* **1973**, *49*, 451–460.
145. Hurst, C.J. Presence of enteric viruses in freshwater and their removal by the conventional drinking water treatment process. *Bull. World Health Organ.* **1991**, *69*, 113–119. [PubMed]
146. Templeton, M.R.; Andrews, R.C.; Hofmann, R. Particle-Associated Viruses in Water: Impacts on Disinfection Processes. *Crit. Rev. Environ. Sci. Technol.* **2008**, *38*, 137–164. [CrossRef]
147. Chahal, C.; Van Den Akker, B.; Young, F.; Franco, C.; Blackbeard, J.; Monis, P. Pathogen and Particle Associations in Wastewater: Significance and Implications for Treatment and Disinfection Processes. *Adv. Appl. Microbiol.* **2016**, *97*, 63–119. [PubMed]
148. Characklis, G.W.; Dilts, M.J.; Simmons, O.D.; Likirdopulos, C.A.; Krometis, L.-A.H.; Sobsey, M.D. Microbial partitioning to settleable particles in stormwater. *Water Res.* **2005**, *39*, 1773–1782. [CrossRef] [PubMed]
149. Cizek, A.R.; Characklis, G.W.; Krometis, L.-A.; Hayes, J.A.; Simmons, O.D.; Di Lonardo, S.; Alderisio, K.A.; Sobsey, M.D. Comparing the partitioning behavior of Giardia and Cryptosporidium with that of indicator organisms in stormwater runoff. *Water Res.* **2008**, *42*, 4421–4438. [CrossRef]
150. Stumm, W.; Morgan, J.J. *Aquatic Chemistry: Chemical Equilibria and Rates in Natural Waters*; John Wiley & Sons: Hoboken, NJ, USA, 2012; 1040p.
151. Van Loosdrecht, M.C.; Lyklema, J.; Norde, W.; Schraa, G.; Zehnder, A.J. Electrophoretic mobility and hydrophobicity as a measured to predict the initial steps of bacterial adhesion. *Appl. Environ. Microbiol.* **1987**, *53*, 1898–1901. [CrossRef]
152. Bradford, S.A.; Morales, V.L.; Zhang, W.; Harvey, R.W.; Packman, A.I.; Mohanram, A.; Welty, C. Transport and Fate of Microbial Pathogens in Agricultural Settings. *Crit. Rev. Environ. Sci. Technol.* **2013**, *43*, 775–893. [CrossRef]
153. Krasowska, A.; Sigler, K. How microorganisms use hydrophobicity and what does this mean for human needs? *Front. Cell. Infect. Microbiol.* **2014**, *4*, 112. [CrossRef]
154. Armanious, A.; Aeppli, M.; Jacak, R.; Refardt, D.; Sigstam, T.; Kohn, T.; Sander, M. Viruses at Solid–Water Interfaces: A Systematic Assessment of Interactions Driving Adsorption. *Environ. Sci. Technol.* **2016**, *50*, 732–743. [CrossRef]
155. Hejkal, T.W.; Wellings, F.M.; Lewis, A.L.; LaRock, P.A. Distribution of viruses associated with particles in waste water. *Appl. Environ. Microbiol.* **1981**, *41*, 628–634. [CrossRef]
156. Davies, C.; Yousefi, Z.; Bavor, H. Occurrence of coliphages in urban stormwater and their fate in stormwater management systems. *Lett. Appl. Microbiol.* **2003**, *37*, 299–303. [CrossRef]

157. Jeng, H.C.; England, A.J.; Bradford, H.B. Indicator Organisms Associated with Stormwater Suspended Particles and Estuarine Sediment. *J. Environ. Sci. Health Part A* **2005**, *40*, 779–791. [CrossRef]
158. Kaucner, C.; Davies, C.; Ferguson, C.; Ashbolt, N. Evidence for the existence of Cryptosporidium oocysts as single entities in surface runoff. *Water Sci. Technol.* **2005**, *52*, 199–204. [CrossRef]
159. Shapiro, K.; Miller, W.A.; Silver, M.W.; Odagiri, M.; Largier, J.L.; Conrad, P.A.; Mazet, J.A.K. Research Commentary: Association of Zoonotic Pathogens with Fresh, Estuarine, and Marine Macroaggregates. *Microb. Ecol.* **2012**, *65*, 928–933. [CrossRef]
160. Qualls, R.G.; Flynn, M.P.; Johnson, J.D. The Role of Suspended Particles in Ultraviolet Disinfection. *J. Water Pollut. Control Fed.* **1983**, *55*, 1280–1285.
161. Brettar, I.; Höfle, M.G. Influence of ecosystematic factors on survival of Escherichia coli after large-scale release into lake water mesocosms. *Appl. Environ. Microbiol.* **1992**, *58*, 2201–2210. [CrossRef]
162. Bitton, G.; Fraxedas, R.; Gifford, G. Effect of solar radiation on poliovirus: Preliminary experiments. *Water Res.* **1979**, *13*, 225–228. [CrossRef]
163. Hejkal, T.W.; Wellings, F.M.; LaRock, P.A.; Lewis, A.L. Survival of poliovirus within organic solids during chlorination. *Appl. Environ. Microbiol.* **1979**, *38*, 114–118. [CrossRef]
164. Stagg, C.H.; Wallis, C.; Ward, C.H. Inactivation of clay-associated bacteriophage MS-2 by chlorine. *Appl. Environ. Microbiol.* **1977**, *33*, 385–391. [CrossRef] [PubMed]
165. Sobsey, M.D.; Fuji, T.; Hall, R.M. Inactivation of Cell-Associated and Dispersed Hepatitis A Virus in Water. *J. Am. Water Work. Assoc.* **1991**, *83*, 64–67. [CrossRef]
166. Morita, R.Y. *Bacteria in Oligotrophic Environments: Starvation-Survival Life Styles*; Springer: New York, NY, USA, 1997; 608p.
167. Oliver, J.D. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev.* **2010**, *34*, 415–425. [CrossRef]
168. Justice, S.S.; Hunstad, D.A.; Cegelski, L.; Hultgren, S.J. Morphological plasticity as a bacterial survival strategy. *Nat. Rev. Genet.* **2008**, *6*, 162–168. [CrossRef]
169. Ishihama, A. Adaptation of gene expression in stationary phase bacteria. *Curr. Opin. Genet. Dev.* **1997**, *7*, 582–588. [CrossRef]
170. Hartke, A.; Giard, J.-C.; LaPlace, J.-M.; Auffray, Y. Survival of Enterococcus faecalis in an Oligotrophic Microcosm: Changes in Morphology, Development of General Stress Resistance, and Analysis of Protein Synthesis. *Appl. Environ. Microbiol.* **1998**, *64*, 4238–4245. [CrossRef] [PubMed]
171. Nyström, T. Stationary-Phase Physiology. *Annu. Rev. Microbiol.* **2004**, *58*, 161–181. [CrossRef]
172. Hijnen, W.A.; Dullemont, Y.J.; Schijven, J.F.; Hanzens-Brouwer, A.J.; Rosielle, M.; Medema, G. Removal and fate of Cryptosporidium parvum, Clostridium perfringens and small-sized centric diatoms (Stephanodiscus hantzschii) in slow sand filters. *Water Res.* **2007**, *41*, 2151–2162. [CrossRef]
173. Sofos, J.N. *Detection of Injured Spore-Forming Bacteria from Foods*; CRC Press: Boca Raton, FL, USA, 1989.
174. Oliver, J.D.; Bockian, R. In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of Vibrio vulnificus. *Appl. Environ. Microbiol.* **1995**, *61*, 2620–2623. [CrossRef]
175. Bucheli-Witschel, M.; Bassin, C.; Egli, T. UV-C inactivation in Escherichia coli is affected by growth conditions preceding irradiation, in particular by the specific growth rate. *J. Appl. Microbiol.* **2010**, *109*, 1733–1744. [CrossRef]
176. Lisle, J.T.; Broadaway, S.C.; Prescott, A.M.; Pyle, B.H.; Fricker, C.; McFeters, G.A. Effects of Starvation on Physiological Activity and Chlorine Disinfection Resistance in Escherichia coliO157:H7. *Appl. Environ. Microbiol.* **1998**, *64*, 4658–4662. [CrossRef]
177. Cherchi, C.; Gu, A.Z. Effect of bacterial growth stage on resistance to chlorine disinfection. *Water Sci. Technol.* **2011**, *64*, 7–13. [CrossRef]
178. LaPlace, J.-M.; Thuault, M.; Hartke, A.; Boutibonnes, P.; Auffray, Y. Sodium hypochlorite stress in Enterococcus faecalis: Influence of antecedent growth conditions and induced proteins. *Curr. Microbiol.* **1997**, *34*, 284–289. [CrossRef]
179. Hiatt, C.W. Kinetics of the inactivation of viruses. *Bacteriol. Rev.* **1964**, *28*, 150–163. [CrossRef]
180. Cerf, O. A Review Tailing of Survival Curves of Bacterial Spores. *J. Appl. Bacteriol.* **1977**, *42*, 1–19. [CrossRef] [PubMed]
181. Haas, C.N.; Karra, S.B. Kinetics of microbial inactivation by chlorine—I Review of results in demand-free systems. *Water Res.* **1984**, *18*, 1443–1449. [CrossRef]
182. Haas, C.N.; Joffe, J.; Anmangandla, U.; Jacangelo, J.G.; Heath, M. Water quality and disinfection kinetics. *J. Am. Water Works Assoc.* **1996**, *88*, 95–103. [CrossRef]
183. McFeters, G.A. Detection and significance of injured indicator and pathogenic bacteria in water. In *Injured Index and Pathogenic Bacteria: Occurrence and Detection in Foods, Water, and Feed*; CRC Press: Boca Raton, FL, USA, 1989; pp. 179–210.
184. Ray, B. *Injured Index and Pathogenic Bacteria: Occurrence and Detection in Foods, Water and Feeds*; CRC Press: Boca Raton, FL, USA, 1989; 240p.
185. Figueras, M.J.; Borrego, J.J. New Perspectives in Monitoring Drinking Water Microbial Quality. *Int. J. Environ. Res. Public Health* **2010**, *7*, 4179–4202. [CrossRef]
186. Saxena, G.; Bharagava, R.N.; Kaithwas, G.; Raj, A. Microbial indicators, pathogens and methods for their monitoring in water environment. *J. Water Health* **2015**, *13*, 319–339. [CrossRef]
187. McFeters, G.A. Enumeration, Occurrence, and Significance of Injured Indicator Bacteria in Drinking Water. In *Drinking Water Microbiology*; Springer: New York, NY, USA, 1990; pp. 478–492.

188. Andrews, W.H. Importance and regulatory implication of the recovery of injured microorganisms from foods and water. In *Injured Index and Pathogenic Bacteria: Occurrence and Detection in Foods, Water and Feeds*; CRC Press: Boca Raton, FL, USA, 1989.
189. Pommepuy, M.; Butin, M.; Derrien, A.; Gourmelon, M.; Colwell, R.R.; Cormier, M. Retention of enteropathogenicity by viable but nonculturable Escherichia coli exposed to seawater and sunlight. *Appl. Environ. Microbiol.* **1996**, *62*, 4621–4626. [CrossRef]
190. Rahman, I.; Shahamat, M.; Chowdhury, M.A.; Colwell, R.R. Potential virulence of viable but nonculturable Shigella dysenteriae type 1. *Appl. Environ. Microbiol.* **1996**, *62*, 115–120. [CrossRef]
191. Simon, E.M.; Stahl, K.L.; Wilson, J.B. Preservation by freeze-drying and the stability of virulence of Salmonella typhimurium. *Appl. Microbiol.* **1963**, *11*, 371–376. [CrossRef]
192. Sorrells, K.M.; Speck, M.L.; Warren, J.A. Pathogenicity of Salmonella gallinarum after metabolic injury by freezing. *Appl. Microbiol.* **1970**, *19*, 39–43. [CrossRef]
193. Xu, H.-S.; Roberts, N.; Singleton, F.L.; Attwell, R.W.; Grimes, D.J.; Colwell, R.R. Survival and viability of nonculturableEscherichia coli andVibrio cholerae in the estuarine and marine environment. *Microb. Ecol.* **1982**, *8*, 313–323. [CrossRef]
194. Pinto, D.; Almeida, V.; Santos, M.A.; Chambel, L. Resuscitation of Escherichia coli VBNC cells depends on a variety of environmental or chemical stimuli. *J. Appl. Microbiol.* **2011**, *110*, 1601–1611. [CrossRef] [PubMed]
195. Nyström, T. Nonculturable bacteria: Programmed survival forms or cells at death's door? *Bioessays* **2003**, *25*, 204–211. [CrossRef] [PubMed]
196. Bogosian, G.; Morris, P.J.L.; O'Neil, J.P. A Mixed Culture Recovery Method Indicates that Enteric Bacteria Do Not Enter the Viable but Nonculturable State. *Appl. Environ. Microbiol.* **1998**, *64*, 1736–1742. [CrossRef]
197. Bogosian, G.; Bourneuf, E.V. A matter of bacterial life and death. *EMBO Rep.* **2001**, *2*, 770–774. [CrossRef]
198. Dukan, S.; Lévi, Y.; Touati, D. Recovery of culturability of an HOCl-stressed population of Escherichia coli after incubation in phosphate buffer: Resuscitation or regrowth? *Appl. Environ. Microbiol.* **1997**, *63*, 4204–4209. [CrossRef]
199. Kell, D.B.; Kaprelyants, A.S.; Weichart, D.H.; Harwood, C.R.; Barer, M.R. Viability and activity in readily culturable bacteria: A review and discussion of the practical issues. *Antonie Leeuwenhoek* **1998**, *73*, 169–187. [CrossRef]
200. Barer, M.R.; Harwood, C.R. Bacterial Viability and Culturability. *Adv. Microb. Physiol.* **1999**, *41*, 93–137. [CrossRef]
201. Oliver, J.D. The viable but nonculturable state in bacteria. *J. Microbiol.* **2005**, *43*, 93–100.
202. Kapuscinski, R.B.; Mitchell, R. Solar radiation induces sublethal injury in Escherichia coli in seawater. *Appl. Environ. Microbiol.* **1981**, *41*, 670–674. [CrossRef]
203. Calabrese, J.P.; Bissonnette, G.K. Improved membrane filtration method incorporating catalase and sodium pyruvate for detection of chlorine-stressed coliform bacteria. *Appl. Environ. Microbiol.* **1990**, *56*, 3558–3564. [CrossRef]
204. Al-Qadiri, H.M.; Al-Alami, N.I.; Al-Holy, M.A.; Rasco, B.A. Using Fourier Transform Infrared (FT-IR) Absorbance Spectroscopy and Multivariate Analysis To Study the Effect of Chlorine-Induced Bacterial Injury in Water. *J. Agric. Food Chem.* **2008**, *56*, 8992–8997. [CrossRef] [PubMed]
205. Tandon, P.; Chhibber, S.; Reed, R. The enumeration of chlorine-injured Escherichia coli and Enterococcus faecalis is enhanced under conditions where reactive oxygen species are neutralized. *Lett. Appl. Microbiol.* **2007**, *44*, 73–78. [CrossRef] [PubMed]
206. Cho, M.; Kim, J.; Kim, J.Y.; Yoon, J.; Kim, J.-H. Mechanisms of Escherichia coli inactivation by several disinfectants. *Water Res.* **2010**, *44*, 3410–3418. [CrossRef] [PubMed]
207. Hong, R.; Kang, T.Y.; Michels, C.A.; Gadura, N. Membrane Lipid Peroxidation in Copper Alloy-Mediated Contact Killing of Escherichia coli. *Appl. Environ. Microbiol.* **2012**, *78*, 1776–1784. [CrossRef]
208. Domek, M.J.; Lechevallier, M.W.; Cameron, S.C.; McFeters, G.A. Evidence for the role of copper in the injury process of coliform bacteria in drinking water. *Appl. Environ. Microbiol.* **1984**, *48*, 289–293. [CrossRef]
209. Yamanaka, M.; Hara, K.; Kudo, J. Bactericidal Actions of a Silver Ion Solution on Escherichia coli, Studied by Energy-Filtering Transmission Electron Microscopy and Proteomic Analysis. *Appl. Environ. Microbiol.* **2005**, *71*, 7589–7593. [CrossRef]
210. Nelson, K.L.; Boehm, A.B.; Davies-Colley, R.J.; Dodd, M.C.; Kohn, T.; Linden, K.G.; Liu, Y.; Maraccini, P.A.; McNeill, K.; Mitch, W.A.; et al. Sunlight-mediated inactivation of health-relevant microorganisms in water: A review of mechanisms and modeling approaches. *Environ. Sci. Process. Impacts* **2018**, *20*, 1089–1122. [CrossRef]
211. Barr, J.N.; Fearns, R. How RNA viruses maintain their genome integrity. *J. Gen. Virol.* **2010**, *91*, 1373–1387. [CrossRef]
212. Olsthoorn, R.C.; Van Duin, J. Evolutionary reconstruction of a hairpin deleted from the genome of an RNA virus. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 12256–12261. [CrossRef]
213. Gmyl, A.P.; Belousov, E.V.; Maslova, S.V.; Khitrina, E.V.; Chetverin, A.B.; Agol, V.I. Nonreplicative RNA Recombination in Poliovirus. *J. Virol.* **1999**, *73*, 8958–8965. [CrossRef]
214. Oguma, K.; Katayama, H.; Ohgaki, S. Photoreactivation of Escherichia coli after Low- or Medium-Pressure UV Disinfection Determined by an Endonuclease Sensitive Site Assay. *Appl. Environ. Microbiol.* **2002**, *68*, 6029–6035. [CrossRef]
215. Zimmer, J.L.; Slawson, R.M. Potential Repair of Escherichia coli DNA following Exposure to UV Radiation from Both Medium- and Low-Pressure UV Sources Used in Drinking Water Treatment. *Appl. Environ. Microbiol.* **2002**, *68*, 3293–3299. [CrossRef]
216. Morita, S.; Namikoshi, A.; Hirata, T.; Oguma, K.; Katayama, H.; Ohgaki, S.; Motoyama, N.; Fujiwara, M. Efficacy of UV Irradiation in Inactivating Cryptosporidiumparvum Oocysts. *Appl. Environ. Microbiol.* **2002**, *68*, 5387–5393. [CrossRef]
217. Kim, J.Y.; Lee, C.; Sedlak, D.L.; Yoon, J.; Nelson, K.L. Inactivation of MS2 coliphage by Fenton's reagent. *Water Res.* **2010**, *44*, 2647–2653. [CrossRef]

218. Nieto-Juarez, J.I.; Kohn, T. Virus removal and inactivation by iron (hydr)oxide-mediated Fenton-like processes under sunlight and in the dark. *Photochem. Photobiol. Sci.* **2013**, *12*, 1596–1605. [CrossRef]
219. American Public Health Association. *Standard Methods: For the Examination of Water and Wastewater*, 18th ed.; American Public Health Association: Washington, DC, USA, 1992; 1000p.
220. McFeters, G.A.; Cameron, S.C.; Lechevallier, M.W. Influence of diluents, media, and membrane filters on detection fo injured waterborne coliform bacteria. *Appl. Environ. Microbiol.* **1982**, *43*, 97–103. [CrossRef]
221. Dutka, B.J.; Jackson, M.J.; Bell, J.B. Comparison of Autoclave and Ethylene Oxide-Sterilized Membrane Filters Used in Water Quality Studies. *Appl. Microbiol.* **1974**, *28*, 474–480. [CrossRef]
222. Lin, S.D. Membrane filter method for recovery of fecal coliforms in chlorinated sewage effluents. *Appl. Environ. Microbiol.* **1976**, *32*, 547–552. [CrossRef]
223. Yakub, G.P.; Castric, D.A.; Stadterman-Knauer, K.L.; Tobin, M.J.; Blazina, M.; Heineman, T.N.; Yee, G.Y.; Frazier, L. Evaluation of Colilert and Enterolert Defined Substrate Methodology for Wastewater Applications. *Water Environ. Res.* **2002**, *74*, 131–135. [CrossRef]
224. Ko, G.; Iii, O.D.S.; Likirdopulos, C.A.; Worley-Davis, L.; Williams, M.; Sobsey, M.D. Investigation of Bioaerosols Released from Swine Farms using Conventional and Alternative Waste Treatment and Management Technologies. *Environ. Sci. Technol.* **2008**, *42*, 8849–8857. [CrossRef]
225. McCrady, M.H. Standard methods for the examination of water and waste-water. *Am. J. Public Health Nations Health* **1966**, *56*, 684. [CrossRef]
226. Wu, V.C.H. Erratum to "A review of microbial injury and recovery methods in food". *Food Microbiol.* **2008**, *25*, 1001. [CrossRef]
227. Ngwa, G.; Schop, R.; Weir, S.; León-Velarde, C.; Odumeru, J. Detection and enumeration of E. coli O157:H7 in water samples by culture and molecular methods. *J. Microbiol. Methods* **2013**, *92*, 164–172. [CrossRef] [PubMed]
228. Begley, M.; Gahan, C.G.; Hill, C. The interaction between bacteria and bile. *FEMS Microbiol. Rev.* **2005**, *29*, 625–651. [CrossRef]
229. Lechevallier, M.W.; Cameron, S.C.; McFeters, G.A. New medium for improved recovery of coliform bacteria from drinking water. *Appl. Environ. Microbiol.* **1983**, *45*, 484–492. [CrossRef]
230. McDonald, L.C.; Hackney, C.R.; Ray, B. Enhanced recovery of injured Escherichia coli by compounds that degrade hydrogen peroxide or block its formation. *Appl. Environ. Microbiol.* **1983**, *45*, 360–365. [CrossRef]
231. Nebra, Y.; Jofre, J.; Blanch, A.R. The effect of reducing agents on the recovery of injured Bifidobacterium cells. *J. Microbiol. Methods* **2002**, *49*, 247–254. [CrossRef]
232. Casanova, L.M.; Walters, A.; Nagahawatte, A.; Sobsey, M.D. Ceramic pot filter user satisfaction and water quantity production in tsunami-affected Sri Lankan communities. *J. Water Sanit. Hyg. Dev.* **2013**, *3*, 646–648. [CrossRef]
233. Kaufman, A.R.; Casanova, L.M.; Sobsey, M.D. Efficacy of a ceramic siphon household water filter for removal of pathogenic microorganisms: Lifespan volume test. *J. Water Sanit. Hyg. Dev.* **2011**, *1*, 102–111. [CrossRef]
234. Elliott, M.; Stauber, C.; Koksal, F.; DiGiano, F.; Sobsey, M. Reductions of E. coli, echovirus type 12 and bacteriophages in an intermittently operated household-scale slow sand filter. *Water Res.* **2008**, *42*, 2662–2670. [CrossRef]
235. Elliott, M.; DiGiano, F.; Sobsey, M. Virus attenuation by microbial mechanisms during the idle time of a household slow sand filter. *Water Res.* **2011**, *45*, 4092–4102. [CrossRef]
236. Wright, J.; Gundry, S.; Conroy, R.M. Household drinking water in developing countries: A systematic review of microbiological contamination between source and point-of-use. *Trop. Med. Int. Health* **2004**, *9*, 106–117. [CrossRef]
237. Oswald, W.E.; Bern, C.; Cabrera, L.; Lescano, A.G.; Gilman, R.H.; Calderon, M.M. Fecal Contamination of Drinking Water within Peri-Urban Households, Lima, Peru. *Am. J. Trop. Med. Hyg.* **2007**, *77*, 699–704. [CrossRef]
238. Rufener, S.; Mäusezahl, D.; Mosler, H.-J.; Weingartner, R. Quality of Drinking-water at Source and Point-of-consumption—Drinking Cup as a High Potential Recontamination Risk: A Field Study in Bolivia. *J. Health Popul. Nutr.* **2010**, *28*, 34–41. [CrossRef]
239. Günther, I.; Schipper, Y. Pumps, germs and storage: The impact of improved water containers on water quality and health. *Health Econ.* **2012**, *22*, 757–774. [CrossRef]
240. Holt, K.; Ortiz, G.M.; Sobsey, M.D.; Stauber, C.E. An Examination of Household Drinking Water Storage and Management Practices in Bonao, Dominican Republic from September 2005–January 2006. *Am. J. Trop. Med. Hyg.* **2010**, *83*, 70–140.
241. Hamoudi, A.; Jeuland, M.; Lombardo, S.; Patil, S.; Pattanayak, S.K.; Rai, S. The Effect of Water Quality Testing on Household Behavior: Evidence from an Experiment in Rural India. *Am. J. Trop. Med. Hyg.* **2012**, *87*, 18–22. [CrossRef]
242. Lantagne, D.S.; Clasen, T.F. Use of Household Water Treatment and Safe Storage Methods in Acute Emergency Response: Case Study Results from Nepal, Indonesia, Kenya, and Haiti. *Environ. Sci. Technol.* **2012**, *46*, 11352–11360. [CrossRef]
243. Lechevallier, M.W.; Norton, W.D.; Lee, R.G. Occurrence of Giardia and Cryptosporidium spp. in surface water supplies. *Appl. Environ. Microbiol.* **1991**, *57*, 2610–2616. [CrossRef]
244. Dugan, N.R.; Fox, K.R.; Owens, J.H.; Miltner, R.J. Controlling Cryptosporidium oocysts Using Conventional Treatment. *J. Am. Water Works Assoc.* **2001**, *93*, 64–76. [CrossRef]
245. Lechevallier, M.W.; Norton, W.D. Giardia and Cryptosporidium in raw and finished water. *J. Am. Water Works Assoc.* **1995**, *87*, 54–68. [CrossRef]

246. United States Environmental Protection Agency. *Method 1604: Total Coliforms and Escherichia coli in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium)*; United States Environmental Protection Agency: Washington, DC, USA, 2002.
247. Bowman, W.; Messner, M.; Regli, S.; Bender, J. Measuring the effectiveness of performance-based training. *J. Water Health* **2008**, *7*, 155–167. [CrossRef] [PubMed]
248. Izard, D.; Ferragut, C.; Gavini, F.; Kersters, K.; De Ley, J.; Leclerc, H. Klebsiella terrigena, a New Species from Soil and Water. *Int. J. Syst. Bacteriol.* **1981**, *31*, 116–127. [CrossRef]