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Microbial ecology of drinking water distribution systems

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The supply of clean drinking water is a major, and relatively recent, public health milestone. Control of microbial growth in drinking water distribution systems, often achieved through the addition of disinfectants, is essential to limiting waterborne illness, particularly in immunocompromised subpopulations. Recent inquiries into the microbial ecology of distribution systems have found that pathogen resistance to chlorination is affected by microbial community diversity and interspecies relationships. Research indicates that multispecies biofilms are generally more resistant to disinfection than single-species biofilms. Other recent findings are the increased survival of the bacterial pathogen *Legionella pneumophila* when present inside its protozoan host *Hartmannella vermiformis* and the depletion of chloramine disinfectant residuals by nitrifying bacteria, leading to increased overall microbial growth. Interactions such as these are unaccounted for in current disinfection models. An understanding of the microbial ecology of distribution systems is necessary to design innovative and effective control strategies that will ensure safe and high-quality drinking water.

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Introduction

Many problems in drinking water distribution systems (DSs) are microbial in nature, including biofilm growth [1], nitrification [2••], microbially mediated corrosion [3], and the persistence of pathogens [4•]. While documented epidemiological studies that directly link disease occurrence with the level of DS pathogens are scarce, waterborne pathogens that are able to persist and reproduce in the DS can cause infections of the gastrointestinal tract, skin and lymph nodes [5]. The DS pathogens comprising the *Mycobacterium avium* complex, for example, have been identified as the most common source of bacterial infection in AIDS patients [6]. The conventional approach to biological control in DSs (i.e. maintaining a disinfectant residual) is often ineffective at controlling microbial growth [7]. Our understanding of the mechanisms of microbial growth in the presence of disinfectants is superficial, and studying the microbial ecology of DSs will continue to provide needed insights to help resolve public health concerns associated with microbial growth in these engineered systems.

In this paper, we describe the importance of biofilm processes in DSs. We then review the state of knowledge of microbial community diversity in DSs, with a focus on nitrifiers, bacterial pathogens, and relationships between bacterial pathogens and protozoa. We review complexities associated with controlling microbial growth and also discuss monitoring and modeling strategies used to improve our understanding of biological processes in DSs. Owing to the abundance of literature on DSs and the availability of relevant reviews (e.g. [5]), we have narrowed the scope of this review to studies on microbial ecology and microorganisms in real and model DSs published primarily during the past three years.

The importance of biofilms

Biofilms are suspected to be the primary source of microorganisms in DSs that are fed adequately treated water and have no pipeline breaches [8] and are a particular concern in older DSs [9]. In a recent study of DSs in Parisian suburbs, it was found that biofilms attached to the surface of a 100 mm diameter pipe contained 25 times more bacterial cells per unit length than the adjacent bulk water [10]. Biofilms predominate because attached cells have certain advantages over planktonic cells, such as the ability to metabolize recalcitrant organic compounds [1] and increased resistance to chlorine and other biocides [4•,11]. Disinfection with chlorine dioxide and chlorite, for example, can reduce the concentration of planktonic bacteria, but have little to no effect on the concentration of biofilm bacteria [12]. The mechanism behind the observed resistance of biofilm cells to disinfection is unknown, although hypotheses include mass transfer resistance [13], the formation of persister cells [14], and protection owing to the production of extracellular polymeric substances [15]. The history of disinfection in DSs can also influence biofilm growth. Lapses in chlorination can lead to regrowth of biofilm communities and increased resistance of biofilm bacteria to chlorine [16•]. Such findings implicate the importance of maintaining a continuous disinfectant residual in DSs.

Microbial community diversity

Information on the microbial community diversity of DSs is scant, because molecular microbial ecology tools have not yet been used widely in this field. Moreover, opportunities to sample biofilms from real DSs are limited. Therefore, many studies have used surrogates such as model DSs and removable coupons for biofilm attachment inserted (for short times) in real DSs. Limitations with such studies are illustrated in a long-term (three year) study of a model DSs [17••]. In this study, it was found by 16S rRNA gene sequence analysis that biofilm species richness was comparable to the species richness in the bulk water during the initial stages of biofilm formation owing to the attachment of bulk water cells, and then decreased as a dominant bacterium related to *Nitrospira* colonized the surfaces — comprising 78% of the biofilm cells. Biofilm species richness increased again as a stable biofilm community composition was achieved after almost two years [17••]. This work suggests that biofilm development may require several years before steady-state is achieved, which limits the relevance of short-term model studies [17••]. Consistent with this observation, other studies with model DSs suggest that as biofilms age, cell density stabilizes and species diversity increases [18].

Researchers who have started the process of characterizing microbial diversity in DSs have isolated several novel bacterial strains from municipal DSs [19,20]. In most cases, a rigorous characterization of these strains is still incomplete. A recent analysis of the bulk water of a chlorinated DS found that Gram-positive bacteria and *Alpha-*, *Beta-* and *Gammaproteobacteria* constituted the major groups among heterotrophic isolates [21]. *Alphaproteobacteria* were the dominant isolates in both chloraminated and chlorinated water from model DSs, whereas *Betaproteobacteria* were found to be more abundant in chloraminated water than in chlorinated water [22]. 16S rRNA gene-directed PCR and denaturing gradient gel electrophoresis (DGGE) revealed that *Betaproteobacteria* were also abundant in biofilms of non-chlorinated DSs [4•]. These studies indicate that microbial community diversity is impacted by the disinfection strategy. There is also evidence that diversity can affect disinfection efficacy and pathogen survival. For example, recent work with a flow cell system showed that multispecies biofilms were more resistant to biocides than single-species biofilms [23]. The specific mechanism for this is unknown, but a more complete picture of microbial community diversity and interspecies relationships should facilitate a better understanding of disinfection resistance phenomena.

Nitrifiers

Nitrifying organisms, belonging primarily to the *Alpha-*, *Beta-* and *Gammaproteobacteria*, have been the subject of several DS studies because nitrification can contribute to the depletion of monochloramine and results in the formation of nitrate [24]. *Nitrosomonas* spp., members of the *Betaproteobacteria*, were identified using 16S rRNA gene-targeted terminal restriction fragment length polymorphism (T-RFLP) and sequencing of ammonia monooxygenase genes as dominant ammonia-oxidizing bacteria (AOB) in biofilm and bulk water samples from pilot- and full-scale DS studies [2••,24,25]. Another subgroup of *Betaproteobacteria*, *Nitrosospira* spp., was found to constitute a small fraction of the AOB in these systems [2••,24,25]. The use of 16S rRNA gene-directed PCR and DGGE also confirmed the presence of both *Nitrosomonas* spp. and *Nitrosospira* spp. in DS bulk water and biofilms [26]. *Nitrospira* spp. were identified in several studies as the dominant nitrite-oxidizing bacteria (NOB) in bulk water and biofilms using 16S rRNA gene clone libraries [27] and 16S rRNA gene-targeted T-RFLP [2••,24]. *Nitrobacter* spp., NOB belonging to the *Alphaproteobacteria*, were also detected in biofilms of chloraminated drinking water [2••].

Nitrification processes can be very important to DS management strategies, because they affect the chloramine residual. In a comparison of chlorinated and chloraminated distribution systems, losses in chloramine level owing to nitrification (measured by the increase in nitrate) led to increased overall microbial growth, as determined by heterotrophic plate counts [28]. It appears that AOB are present in chloraminated systems irrespective of temperature fluctuations and can be controlled only through very high or very low chloramine levels, because of the disinfection action at high levels and the scarcity of ammonia at low levels [29•].

Pathogens

The persistence and growth of pathogens is a central concern in DSs. Field surveys using PCR and Southern blot hybridization reported the regular detection of pathogens, including *Legionella* spp. and atypical mycobacteria [4•]. *Cryptosporidium* spp. oocysts were detected in bulk water samples [30] and *Helicobacter* spp. were identified in biofilms [31] in DSs using nested PCR methods. Multiplex PCR analysis was used to detect *Mycobacterium avium* and *Mycobacterium intracellulare* as well as several other *Mycobacterium* spp. in water column and biofilm samples [32]. *Aeromonas* spp. have also been found in DSs and PCR-based methods were used to quantify the abundance of specific virulence factor genes in isolated *Aeromonas* strains in drinking water [33]. In addition to the detection of specific pathogens and virulence factors, one study monitored antibiotic resistance genes in DS biofilms. Using PCR-based methods, resistance genes responsible for vancomycin-resistance (*van*A) and for β-lactamase activities (*amp*C) were detected in DS biofilms [34].

Besides bacterial and protozoan pathogens, viral pathogens also persist in DSs; for example, enteroviruses and

adenoviruses have been found (reviewed in [35]). As many pathogenic viruses are known to be stable in the environment and are resistant to conventional inactivation methods [36], it is clear that more research is necessary to understand the role of pathogenic viruses in DS-related waterborne illnesses.

The use of molecular tools to detect pathogens in drinking water systems, including PCR-based methods, DNA- and RNA-targeted hybridizations, and microarray-based technologies, allows for a much more sensitive detection of pathogens than was previously possible with culture-based methods (reviewed in [37]). Although several studies have begun to apply these tools to study DS management strategies, a substantial number of studies in this area are still performed with conventional culture-based techniques. Two recent examples of the application of molecular techniques in DS management research are noteworthy. A flow chamber study verified that the presence of high concentrations of disinfectants was not sufficient to eliminate the survival of pathogens, including *Legionella pneumophila* and *Escherichia coli* [38]. Similarly, another study found that the application of two common disinfectants, monochloramine and ultra-violet light, did not deter *L. pneumophila* from accumulating in biofilms in a pilot-scale DS [39]. More conventional studies in this area found that biofilms exposed to strains of *E. coli* and *Klebsiella pneumoniae* developed stable populations of both opportunistic pathogens proportional to the biofilm density of heterotrophic bacteria [40]. Likewise, *Mycobacterium xenopi* was found to colonize drinking water biofilms [41]. It was further determined that *M. xenopi* exhibited long-term persistence and that a steady concentration of *M. xenopi* cells was returned to the water column from biofilms [41]. Irrespective of the techniques used, these studies highlight the danger of pathogen survival in biofilms: pathogens in biofilms are protected from disinfection and are being released to the bulk water used for human consumption.

Bacterial pathogen–protozoon interactions

Studying the ecology of bacterial pathogen–protozoon interactions might help to improve our understanding of the persistence of bacterial pathogens in drinking water. For example, it has been estimated that the amoeba *Acanthamoeba polyphaga* can contain between 1 to 120 *M. avium* cells and can host even higher levels of *L. pneumophila* [42]. An inactivation study for the bacterial pathogen *Burkholderia pseudomallei* found that co-culture with the amoeba *Acanthamoeba astronyxis* increased the resistance of *B. pseudomallei* to disinfection, requiring 100 times more monochloramine to achieve similar disinfectant efficacy than when cultured alone [43]. Additionally, the depletion of disinfectant can result in a re-colonization of biofilms by bacterial pathogens, such as *L. pneumophila*, protected in amoeba [44]. *L. pneumophila* has been found to proliferate in drinking water biofilms in the presence of the protozoon *Hartmannella vermiformis*, and after 14 days of co-culture intracellular growth was found in 90% of the protozoa [45•]. There is also evidence that intracellular growth selects for virulence factors that affect pathogenesis in protozoon hosts (reviewed in [46]).

Complexities associated with controlling microbial growth

Optimizing the management of DSs and controlling microbial growth is difficult because of the complexity of these systems. The survival of microorganisms is based upon interactions of many variables, including temperature [47], pipe surface [48], nutrient levels [49,50] and type and concentration of disinfectants [51]. Microbial growth can be controlled to some extent through providing a disinfectant residual [52] and by reducing the biodegradable organic matter [53]. Uncontrollable events, such as seasonal fluctuations of precipitation, can lead to even greater complexity. For example, a study of Mexico City's chlorinated DS found that levels of fecal streptococci were significantly higher in the dry season than in the wet season, whereas *H. pylori* levels remained fairly constant through the seasons [54]. This illustrates that uncontrollable complexity such as seasonal precipitation can lead to species-specific responses.

Another, often uncontrollable, complexity is the type of pipe material used in the DS. Certain pipe materials can stimulate growth by releasing iron and phosphorus in their bioavailable forms [55,56] and by neutralizing the disinfectant residual [48,57]. Soft deposits that settle to the pipe floor can be a major source of available nutrients, and the removal of such deposits has been associated with reduced microbial growth [58•]. It should also be noted that the release of some compounds, such as copper from copper pipes, slows biofilm development, presumably because they are toxic or inhibitory to microorganisms [56,59].

Integrating system knowledge: monitoring and modeling

The fundamental biological concern in drinking water supply is to minimize contamination with pathogens. As discussed above, pathogen survival in DSs is based upon complex interactions between physical, chemical and operational factors, and microbial ecology. An important initial step to controlling pathogens is to develop effective monitoring strategies that take the microbial ecology of DSs into account. Culture-based methods often underestimate or distort the community profile because many microorganisms are in a viable but non-culturable state, prompting interest in alternative monitoring methods [26]. Pathogen-specific monitoring could take the form of PCR-based methods, nucleic acid hybridizations or immunological-based methods [37,60,61]. Metagenomic analyses can be used to determine the metabolic and functional potential of entire microbial communities.

A metagenomic approach has already proved useful in determining phylogenetic and functional gene diversity [62], and could be used to further determine the presence of genes conferring virulence [33] and antibiotic resistance [34]. Linking metagenomic approaches with quantitative molecular tools will make it possible to integrate effective monitoring with the control of microbial growth.

The complexity of controlling microbial growth in DSs calls for the use of mathematical models; however, it is challenging to accurately model the processes and interactions in DSs. Multipopulation biofilm models are becoming increasingly complex [63–66], as are models describing biofilm disinfection [14,67] and bacterial regrowth in DSs [68••]. A limitation of current disinfection models is that they almost exclusively include single-population models, with some exceptions [69,70]. The development of multipopulation models of DS biofilms that take into account the effects of disinfectants on microbial ecology will help to determine optimal operational parameters and lead to knowledgeable decisions regarding the management of drinking water supply. The complexity of the DS must then be accounted for by incorporating the multipopulation disinfection model into a large-scale, spatially distributed hydraulic model that integrates knowledge about the layout of the pressurized pipe system, such as the mechanistic model recently developed by DiGiano and Zhang [68••].

Conclusions

It is clear that standard chlorination strategies are sometimes inadequate for controlling regrowth in the DS, and can be improved upon with a better understanding of microbial ecology. Bacterial, protozoon and viral pathogens can resist disinfection through protection within biofilms and resistant host cells. From the viewpoint of environmental biotechnology, this complexity presents a great challenge to providing safe, clean drinking water to the public. Future research will utilize advanced, non-culture-based monitoring techniques to more completely describe pathogen presence in DSs. The elucidation of resistance mechanisms will allow the DS to be modeled accurately and will provide insights into novel control strategies.

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