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Review

Knowledge to Predict Pathogens: *Legionella pneumophila* Lifecycle Critical Review Part I Uptake into Host Cells

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Abstract: *Legionella pneumophila* (*L. pneumophila*) is an infectious disease agent of increasing concern due to its ability to cause Legionnaires' Disease, a severe community pneumonia, and the difficulty in controlling it within water systems. *L. pneumophila* thrives within the biofilm of premise plumbing systems, utilizing protozoan hosts for protection from disinfectants and other environmental stressors. While there is a great deal of information regarding how *L. pneumophila* interacts with protozoa and human macrophages (host for human infection), the ability to use this data in a model to attempt to predict a concentration of *L. pneumophila* in a water system is not known. The lifecycle of *L. pneumophila* within host cells involves three processes: uptake, growth, and egression from the host cell. The complexity of these three processes would risk conflation of the concepts; therefore, this review details the available information regarding how *L. pneumophila* invades host cells (uptake) within the context of data needed to model this process, while a second review will focus on growth and egression. The overall intent of both reviews is to detail how the steps in *L. pneumophila*'s lifecycle in drinking water systems affect human infectivity, as opposed to detailing just its growth and persistence in drinking water systems.

Keywords: *Legionella pneumophila*; legionellosis; uptake process; drinking water; review

1. Introduction

1.1. Rationale: Human Health Impacts and Level of Concern

Legionellosis, a set of two respiratory diseases caused by the inhalation of *Legionella* bacteria, incorporates Pontiac Fever and Legionnaires' disease (LD), a severe form of pneumonia. Pontiac Fever, a self-limiting flu-like illness, is characterized by a respiratory infection lasting two to five days without the development of a pneumonia [1]. Those affected may experience fever, chills, malaise, myalgia, headache, cough, or chest pain, after a typical incubation period of one to two days [1]. The disease was first discovered in Pontiac, Michigan, USA in 1968. At the time of occurrence, the etiological agent of the disease was unable to be identified, with the source of the outbreak surmised to be a toxin due to the speed of symptom onset and lack of etiological agent [2]. It was not until an outbreak of community pneumonia in 1976 that LD was investigated, and *Legionella pneumophila* (*L. pneumophila*) was identified as the causative agent [1,2]. As the etiological agent was discovered because of an outbreakat an American Legion convention in Philadelphia, LD is thusly named. During this outbreak, 29 of the 182 cases were fatal, and a gram-negative bacillus was isolated and determined to be the cause. It was later determined that the same bacterium was the etiological agent of Pontiac Fever [3–5].

LD has similar symptoms to Pontiac Fever with the addition of a severe pneumonia. Though *Legionella* may cause sub-clinical infections, more serious cases of LD can cause long-term symptoms

including easy fatigue, shortness of breath, muscle or joint pain, productive cough, and memory loss [6–8]. LD can have an incubation time ranging from two days to two weeks, depending on the patient's immunocompetence and if antibiotic therapy was administered [8,9]. There is an increased risk of legionellosis in those with a non-municipal water supply, recent residential plumbing repair, electric water heaters (as opposed to gas), smokers, and those who work more than 40 h a week [10–12]. The elderly, males, smokers, those with nosocomial infections, renal disease or immunodeficiency have increased risk of succumbing to the pneumonia [10,13].

Legionellosis is the most common cause of all reported water-associated diseases in the US, and its incidence is steadily increasing [14,15]. The US averages 10,000–15,000 cases per year, and *L. pneumophila* was the cause of half of all reported waterborne disease outbreaks in 2005–2006 [16–18]. Due to the prevalence and seriousness of LD, *L. pneumophila* was added to the United States Waterborne Disease Outbreak Surveillance System in 2001 and the US Environmental Protection Agency (EPA) Candidate Contaminant List (CLL) as an important pathogen. By 2006, *Legionella* spp. was the third most common etiological agent of waterborne disease reported in the United States [19,20]. The majority of cases are community acquired with an unknown source of infection, though it is difficult to accurately report cases associated with travel, due to varying incubation times, illness occurring far from the infection source, and the possibility that symptoms do not require medical attention [21]. Legionellosis can only be spread when the bacterium is aerosolized and respired [22,23]. Sources of legionellosis outbreaks include contaminated hospital bathrooms [24], grocery-store mist machines [25], sprinklers [26], cooling towers [27–29], heating ventilation air conditioning systems (HVAC) [30,31], humidifiers [32], large warm water systems [33], whirlpools [21,34], pools [21] and dental office equipment [35,36].

1.2. Microbiology of Legionella spp.

Legionella spp. describes a gram-negative bacillus bacterium. The genus is widespread in nature, common to fresh surface water, thermal water, and drinking water environments and has been found regularly in soil samples [37–39]. *L. pneumophila* thrive in temperatures 20–50 °C with little to no growth over 50 °C, but have been isolated from hot water with temperatures as high as 66 °C [40,41]. When temperatures reach 70 °C, the bacteria are killed almost instantly [42]. *L. pneumophila* has been shown to be tolerant to sodium chloride solutions up to 1.5% laboratory experiments and isolated in amoeba co-cultures in natural environments of up to 3% sodium chloride [43,44]. The bacterium has an ideal pH of 6.0–8.0. *L. pneumophila* is tolerant of low-nutrient environments but does require iron to survive and thrive [45,46]. In harsh environments, the bacteria can enter a viable but non-culturable (VBNC), state in which it will not replicate, but can be resuscitated in more friendly conditions [47].

The bacteria are facultative intracellular organism that can replicate freely within host cells and utilize the host for protection from environmental stressors. The most common host cells are predating protozoa such as amoeba. *Legionella* that have passed through protozoan hosts are more infective to mammalian macrophages [48]. Horizontal gene transfer between the bacteria and the host cell is at least partially responsible for this increase in infectivity. Over the course of its evolution, *Legionella* has not only acquired genes from eukaryotic host cells, but also upregulates those genes in the stationary phase of growth [49]. *Legionella* has unique lifecycle traits such as its ability to: attract host cells; utilize multiple pathways of infectivity on the same host cell or different host cells as required; and prevent fusion of the lysozyme to the phagosome to survive in the host. The genes and gene complexes responsible for these unique features have been largely identified. Knockdown experiments have been performed to inform the effect of particular genes on the lifecycle of *Legionella* however, with the exception of Bodet et al., an understanding of how environmental stressors affect these genes and in turn *Legionella*'s lifecycle is lacking [50].

1.3. Epidemiology of Legionellosis

There are at least 51 species of *Legionella* and over 60 serogroups. Of these, 18 species have been shown to be pathogenic with the most common cause of legionellosis being *L. pneumophila* [10,51–53]. In the US, *L. pneumophila* serogroup 1 is responsible for 84.2% of cases and serogroups 2–13 are responsible for 7.4%. *Legionella longbeachae* (*L. longbeachae*) is responsible for 3.9% of cases and *Legionella bozemanii* 2.4% of cases. These case contributions are similar in Europe but change significantly in Australia and New Zealand, where *L. pneumophila* serogroup 1 is responsible for 45.7% of cases, and *L. longbeachae* 30.4% of cases [52]. *L. pneumophila* is more commonly found in water, where *L. longbeachae* is more common in soil and associated with gardening and potting soil [54]. *L. longbeachae* has been shown to have enzyme systems that may assist in the degradation of plant material, possibly explaining its soil harbor [55]. Epidemiological patterns of legionellosis differ in these regions too; studies have shown that more than 50% of confirmed cases in New Zealand and Australia were a result of contact with compost [56,57].

1.4. Objectives of this Review Article

Legionella spp. are ubiquitous in aquatic environments with protozoan hosts [58]. The bacteria engage in a symbiotic relationship with protozoa within biofilms where *Legionella* is present, such as premise plumbing, and in large distribution systems [16,59–61]. It is estimated that 95% of the bacterial biomass in drinking water are found in biofilms on the interior of distribution systems [62]. When temperature or flow rate change, the biofilms can slough off the pipes, releasing *Legionella* and allowing for it to be aerosolized when leaving a showerhead, faucet, or plumbing fixture [63]. Outbreaks of legionellosis are still common due to the difficulty in treating these particular bacteria within the water system. It is an unfortunate incident that alveolar macrophages behave in a nearly identical fashion to the natural hosts of *L. pneumophila*, thus making the bacteria easily capable of human infection. Therefore, *L. pneumophila* infections in humans are incidental, since the alveolar macrophages are the secondary hosts. Lau and Ashbolt [64] developed a critical review on the relationship between amoeba and *L. pneumophila* in water systems. However, this leaves a remaining area of knowledge that needs to be examined: the data requirements to model this relationship between amoeba and *L. pneumophila*. More important is the need to model the kinetics of infection of alveolar macrophages exposed to *L. pneumomophila*, thus allowing for a more significant understanding of the realistic health risks from *L. pneumophila*. Additionally, for a complete picture of distribution system risks, the uptake, growth and egression to infect other cells should be modeled. Other review articles discuss health effects, pathology, treatment, transmission, etiology, epidemiology, and infectivity of *L. pneumophila* [9,20,22,23,65–69]. Reviews that discuss growth and survival of *Legionella* either depend on a laboratory-based models, differing significantly from the biofilm environment, or the article focuses on some particular pathway, proteins, or genetics that affect *Legionella*'s lifecycle. However, they do not focus on all of these aspects in one article [46,68,70–75].

There has been no critical review of how this data can be used in a model for these uptake processes. In the case of *L. pneumophila* modeling, these processes and how the genes regulate these processes is a vital portion of modeling a likely concentration in the drinking water systems. Another gap in reviewing the genomic mechanisms of infection in host cells exists, as will become clearer later in the review. These genomic mechanisms are vital to the lifecycle and pathogenesis of *L. pneumophila*. Additionally, since the processes of infection in amoeba and human alveolar macrophages are very similar, this is a rare opportunity to develop a set of models to predict both engineering impacts by predicting concentrations of *L. pneumophila* in water systems and tie this directly with environmental health impacts on the kinetics of growth in alveolar macrophages. Not all data are conducive to modeling; therefore, this review outlines those gaps in the data that preclude use in modeling. As we will see develop in this review, a challenge in understanding *L. pneumophila* is that its characteristics typically considered within the purview of biomedical research are vital in modeling its health risk potential and control in water systems. This is not wholly unique to *L. pneumophila*; however,

attempting to control it without understanding how to model its lifecycle in conjunction with human infectivity risks has the potential to have serious and long-term negative control implications.

This review focuses on the information needed to model the uptake of *Legionella* into host cells, emphasizing the physiological and genetic pathways, and processes, with an emphasis on *L. pneumophila* and *Acanthamoeba* considering their prevalence, and macrophages considering their requirement for human infectivity. This will allow for: (1) a complete picture of the lifecycle of *L. pneumophila* in water systems, (2) movement towards predictive modeling of *L. pneumophila* concentration, (3) prediction of *L. pneumophila* blooms in water systems and (4) improved dose response knowledge in alveolar macrophages in humans.

2. Materials and Methods

Literature Review Method

To determine the mechanisms, proteins, and genes involved in the uptake of *Legionella* to the host cell, an exhaustive literature review was conducted. Google Scholar, PubMed, Web of Science, Bioline International, and PLOS ONE were searched using the terms: "((*Legionella*) OR (*Legionella pneumophila*) OR (*L. pneumophila*) OR (*Legionella longbeachae*) OR (*L. longbeachae*) OR (*Legionella bozemanii*) OR (*L. bozemanii*)) AND ((Human Health Impacts) OR (Symptoms) OR (Infectivity) OR (Uptake) OR (Host Interaction) OR (Genetic Knockout) OR (Disinfection) OR (*Acanthamoeba*) OR (*Acanthamoeba polyphaga*) OR (*Acanthamoeba castellanii*) OR (*A. polyphaga*) OR (*A. castellanii*) OR (Premise Plumbing) OR (Biofilms))." Relevant citations were forward and reverse citation searched and imported into a Zotero library (https://www.zotero.org/alexismraz/items/). Over 1500 papers were reviewed for relevance and 177 papers were included in this review. In order to be included in the review, papers needed to inform *L. pneumophila*'s ability to invade an amoeba or human alveolar macrophage and come from a reputable, peer-reviewed journal. In order for data to be included in this review, the study must have sufficient data points, be validated, and inform *L. pnuemophila's* ability to invade host cells.

3. Results

3.1. Legionella pneumophila Life Cycle

L. pneumophila has evolved alongside predatory eukaryotes, which are phagocytes, cells that predate using some form of phagocytosis. Therefore, *L. pneumophila* has adapted specialized communication with a variety of phagocyte to invade the host [76,77]. As opposed to other bacteria that are predated upon as a sole mechanism of uptake into amoeba, *L. pneumophila* can attract an amoeba and initiate its own uptake as part of its lifecycle. This specialization causes *L. pneumophila* to have less competition with other bacteria common to its environment, such as *Escherichia coli*, *Aeromonas hydrophilia*, *Flavobacterium breve*, and *Pseudomonas aeruginosa* [78]. *L. pneumophila* is known to invade host cells in a variety of forms including traditional phagocytosis, coiling phagocytosis, and pinocytosis, utilizing multiple receptor-mediated pathways. Not only will *L. pneumophila* utilize different methods to infect different species of host, in many cases, the bacteria are capable of utilizing more than one method of invasion on the same host cell [76–78].

Phagocytosis, a key to the lifecycle of *L. pneumophila*, is also its pathogenesis. A critical portion of the pathogenesis process is the genes, and gene complexes *L. pneumophila* uses to secrete morphological structures (e.g., pili) or reactive proteins. For example, the *Dot/Icm* complex associated with the flagella secretes a Type IV protein that deactivates the capsase-1 response in alveolar macrophages to prevent the destruction of *L. pneumophila* after infection [79]. In the case of uptake, *pilE* allows for the secretion of pilin protein, which, in turn, produces the long pili and allows for binding and uptake to the host cell [80]. Amoeba and alveolar macrophages are different in rates of reaction to these processes; additionally, further complicating the matter, murine macrophages do not respond the same as human macrophages [81].

L. pneumophila can utilize either traditional or coiling phagocytosis to invade protozoan or macrophage host cells. Coiling phagocytosis is a rare form of phagocytosis, occurring less than 10% of the time, resulting in the bacteria internalized within the host in a membrane-bound vacuole that does not fuse with lysosomes [81–84]. The complement receptor 3 (CR3) is responsible in part for the mediation of coiling phagocytosis and persists in the phagosomal membrane [85]. CR3 is also responsible for *L. pneumophila*'s attachment to the surface of macrophages [86].

The outer membrane proteins and lipopolysaccharide (LPS) structures play an important role in adhesion and/or uptake of *L. pneumophila* by protozoa. *L. pneumophila* LPS can access classical pathways for entering cells, utilizing antibody–antigen complexes, as described above. In humans, the activation of the classical pathway can be explained by mediation by antibodies of the IgM class present in the normal human serum (NHS) [87]. However, *L. pneumophila* LPS is also able to enter cells (both amoeba and macrophages) via the alternate pathway if the antibodies needed to activate the classical pathway are not present [88]. The complement component (C3) covalently binds to the major outer membrane protein (MOMP) of *L. pneumophila* via the alternate pathway of complement activation. C3 acts as a ligand for complement receptors CR1 and CR3, which mediate phagocytosis of *L. pneumophila* [89].

L. pneumophila relies on a 24-kDa macrophage infectivity proteniator (Mip) protein to efficiently infect both mammalian phagocytic cells and protozoan cells [90,91]. This protein is coded for by the *mip* gene and is thought to be conserved throughout the *Legionella* genus [92–94]. The Mip sequence from 35 *Legionella* species was conserved at the amino acid level 82% to 99% [94]. Mip exhibits a peptidylprolyl *cis/trans* isomerase (PPIase) activity and belongs to the enzyme family of FK506-binding proteins (FKBP), also seen in *Chlamydia trachomatis* and *Trypanosoma cruzi* [95–97]. Amino acids involved in PPIase activity were found to be totally conserved [94]. When the protein is transported through the cytoplasmic membrane, the N-terminal signal sequence is cleaved off, and the protein is found on the surface of the *L. pneumophila* cell, allowing for its infectivity role [98,99].

It has been shown that, for *L. pneumophila* to have its full virulence in higher organisms, such as guinea pigs, both the full-length Mip protein and PPIase activity are necessary [100]. While the Mip protein is important for invasion and establishment within human macrophages and protozoa, it is not necessary for intracellular replication. The Mip protein is repressed directly after the invasion of a monocytic human cell and regains activity after 24 h of intracellular replication. Regaining Mip activity allows for the bacteria to be infective again after replication [101]. The *mip* gene is therefore an important virulence indicator.

L. pneumophila was shown to require actin polymerization and intact microtubule cytoskeleton to invade HeLa cells effectively [102]. Similar results were shown in *Acanthamoeba castellanii* (*A. castellanii)* and *Vermamoeba vermiformis* (*V. vermiformis*) by utilizing cytochalasin D, a microfilament disrupter. However, cytochalasin D did not affect the uptake of *L. pneumophila* by *Acanthamoeba polyphaga* (*A. polyphaga*) [48,76,103]. This further provides evidence that *L. pneumophila* is capable of utilizing various and sometimes redundant pathways to infect host cells.

The *N*-acetyl-D-galactosamin (Gal/GalNAc) lectin receptor pathway and galactose (gal) are utilized by *L. pneumophila* to invade *V. vermiformis*. It is shown that adding gal or GalNAc to an *V. vermiformis* culture will significantly impair *L. pneumophila*'s ability to invade the host cell [77]. It is believed that *V. vermiformis* relies on this pathway heavily, if not exclusively for receptor-mediated pinocytosis of *L. pneumophila*, whereas other protozoa, such as *A. castellanii* and *A. polyphaga*, utilize multiple pathways for *L. pneumophila* uptake [86,104].

It is uncertain what ligand from *L. pneumophila* activates the Gal/GalNAc lectin receptor pathway, but it has been speculated that it is one of the competence and adherence-associated pili (CAP) [105]. CAP is one of two distinct pili on the surface of *L. pneumophila*. It is a Type IV pili involved in adherence to protozoan and mammalian cells [80]. However, the CAP is not exclusively responsible for this attachment. In protozoa, the CAP communicates with the lectin protozoan receptor [84]. This attachment is not strong over the long term, necessitating the rapid uptake processes seen in the lectin

pathway [82,86]. There is redundancy in the genes responsible for pili production in *L. pneumophila*, *pilD* and *pilE* are both responsible for pre-pilin peptidase and result in the expression of Type IV pili [106,107].

L. pneumophila has high uptake rates in *Acanthamoeba*, a genus of amoeba commonly found in freshwater and known as a common host to the bacteria. *A. polyphaga* has an uptake rate of 73–98% of viable *L. pneumophila* cells, while *A. castellanii* has an uptake rate of 96–100% of viable cells [108]. These rates may be partially explained by the self-induced uptake of the bacterium into the host cell. *A. castellanii* phagocytizes *L. pneumophila* through a receptor-mediated system, utilizing the mannose-associated mannose receptor or mannose-binding lectin (MBL) [76]. *L. pneumophila* does not use the MBL solely for protozoan entry; it is also able to activate the MBL for quick entry to pulmonary macrophages in mammals. Interestingly, the MBL is the same receptor that *A. castellanii* uses to bind to the corneal epithelium, causing ocular infections [76]. *L. pneumophila* has a high affinity for the α1-3d-mannobiose-binding site of the mannose receptor from *A. castellanii*, allowing for efficient uptake by the protozoa [76]. *L. pneumophila* has a slightly lower affinity for the d-mannose-binding receptor in *A. castellanii* [109]. This specificity, while known for *A. castellanii*, is most likely inconsistent among the entire *Acanthamoeba* genus. *L. pneumophila* will utilize predominately different pathways when infecting another genus of protozoa.

The differences between uptake of *L. pneumophila* into amoeba and mammalian macrophages are more pronounced than the change in uptake efficiency in varying species of *Acanthamoeba*. Certain genetic knockdowns, such as *rpoS* and *letA* can be avirulent in protozoan hosts like *A. castellanii*, but completely unaffected in human-derived macrophages such as HL60 and THP-1 [110,111]. Furthermore, *L. pneumophila* can only use the Gal/GalNAc pathway, described above to invade protozoan host cells, whereas it can only use the alternate complement receptor pathways and MOMP to invade macrophage [112]. The redundancy of uptake mechanisms and the genetics involved in these mechanisms allows *L. pneumophila* for flexibility in infecting a variety of host cells. *L. pneumophila* even gains an advantage in passing through a protozoan host prior to infect a mammalian macrophage [113].

L. pneumophila that has been grown in *A. castellanii* culture is phenotypically different from *L. pneumophila* grown in standard media. Amoeba grown *L. pneumophila* cells were more invasive than media-grown *L. pneumophila* cells, 100-fold more invasive for epithelial cells and 10-fold for macrophages and amoeba [113]. The amoeba grown bacteria expressed new proteins that may be responsible for the phenotypic differences amongst the stains [113]. Studies have shown that *L. pneumophila* can horizontally transfer genes to and from host cells [49,114], a possible reason for increased infectivity after passage through a host-cell. However, this increase can also be a byproduct of the host cell acting as a unicellular incubator or "biological gym"; therefore, the produced bacteria are more phenotypically complete allowing for optimal infectivity [40].

3.2. Effects of Engineered System Stressors on L. pneumophila

There has been a variety of water treatment options tested to eradicate *L. pneumophila* in water distribution systems including: oxidizing agents such as chlorine, monochloramine, chlorine dioxide, bromine, iodine, ozone, hydrogen peroxide, ultraviolet (UV) radiation, heat, halogenated hydantoins, silver and copper ions, non-oxidizing agents such as aldehydes, amines, heterocyclic ketones, guanidines, thiocyanates, organo-tin compounds, halogenated amides, halogenated glycols, thiocarbamates, and heat [72,115]. While chlorine is commonly used and known to be effective, the continuous and rising number of legionellosis outbreaks causes concern that chlorine is not effective enough in removing *L. pneumophila* from water distribution systems [116–118]. Considering the bacteria's ability to grow within and use biofilms as protection from chlorination, complete eradication cannot be achieved without elevated water temperatures (>60 °C) [119].

Chlorine exists as hypochlorous acid in water in either its neutral form of HOCl or as hypochlorite ion, OCl^- in environments where pH > 7.6. The term "free chlorine" refers to HOCl and OCl^-, though HOCl is known to be more biocidal. Free chlorine inactivates bacteria by adversely affecting their respiratory and transport activities, causing deleterious effects on bacterial membranes, and causing

direct oxidative damage to proteins and nucleotide bases [47,120]. Free-living *L. pneumophila* were killed within 3 min of being exposed to an aqueous solution of 2 mg/L of chlorine, whereas *L. pneumophila* living within biofilms were much more difficult to treat utilizing chlorine [121]. Well-developed biofilms, those 1–2 months old, were able to protect *L. pneumophila* even when exposed to free chlorine at 50 mg/L for 1 h, a concentration much higher than the 5 mg/L recommended by the European Working Group for Legionella Infections [122,123]. The bacteria were able to survive treatment and continue to grow. Newer biofilms, developed in the past 72 h, were unable to protect the bacteria to the same extent, but the treatment was not effective in eradicating *L. pneumophila* from the water distribution system. While no viable colonies were recovered immediately after treatment, colonies began growing again the next day [124].

The event of *L. pneumophila* being viable but non-culturable (VBNC) is fairly common during water treatment processes and has been documented after chlorination or other high levels of oxidative or physical stress, such as ultraviolet radiation and heat, by multiple studies [47,125–127]. It has been theorized that the bacteria become VBNC either because the cell membrane is not permeable at low chlorine levels, and the cells are stressed or injured during treatment rather than inactivated. Or the membrane is permeabilized early, and the hole allows ethidium monoazide bromide to enter the cell and prevent reproduction, but the polymerase chain reaction (PCR) signal is not always reduced [47]. Both heat and chlorination can result in damage to the cell membrane, causing the cell to become VBNC. Interestingly, this affect is mitigated if *L. pneumophila* is in a starvation state, possibly due to the induction of proteins that cross-protect against other stressors [128]. The occurrence of VBNC *L. pneumophila* in water distribution systems may lead to false confidence that the bacteria have been eradicated from the system if presence is determined by a culture method. In other species of bacteria, it has been shown that gene expression can be altered in the VBNC state, typically showing gene supression [129]. However, it has been shown that VBNC cells retain intact genetic material and there are no genetic knockdowns during the VBNC state [130,131]. *L. pneumophila* are able to produce virulence proteins, including the Mip protein when VBNC [132]. Furthermore, VBNC *L. pneumophila* have been shown to infect amoeba and become culturable again [126,133,134]. There is the possibility that VBNC are still infectious to human macrophages, thereby still presenting a public health risk [122,135,136].

Chlorine neutralization can allow for greater persistence of *L. pneumophila* in the biofilm, accelerate the development of the microbial community, and reduce the susceptibility of disinfection in the future [137]. Therefore, it is important with regards to *L. pneumophila*, to ensure that premise plumbing systems do not go through regular periods of chlorine neutralization. Considering the decay of residual chlorine in large premise plumbing systems, these buildings are more likely to have sections of chlorine neutralization, which may also explain further why they are more at risk for a legionellosis outbreak [136].

It is possible that *L. pneumophila* are utilizing host cells that are resistant to chlorine to grow and thrive, or that chlorine is inefficient at penetrating the biofilm and therefore is not reaching the bacteria [122,138]. When chlorine can reach the bacteria, it is capable of altering gene expression. A 2011 study performed on free living *L. pneumophila* showed that non-lethal levels of chlorine caused differential expression of 391 *L. pneumophila* genes. In general, the chlorine treatment induced genes related to the stress response while repressing genes related to virulence [50]. However, it is unclear if chlorine can induce permanent changes to the bacteria, such as genetic knockdowns, a key to reduced uptake and infectivity. Furthermore, genetic expression studies have only been performed in free living bacteria, and not those within the biofilm.

To treat *L. pneumophila* in premise plumbing systems, it is necessary to penetrate the biofilm. Some studies have effectively used shock hyperchlorination, (short period of elevated chlorine dose between 20–50 mg/L), to treat *L. pneumophila* in premise plumbing systems. However, maintaining high levels of chlorine in the water system can have both human health and structural effects [119,135]. Chlorine can be corrosive to the pipes, and its byproducts can cause adverse health effects for humans and the environment.

The negative side effects of chlorine as a disinfectant lead to the use of monochloramine [139]. Monochloramine (NH_2Cl), a derivative of ammonia, has been shown to be more effective than free chlorine as a residual disinfectant. Hospitals using free chlorine had an odds ratio of 10.2 of an LD outbreak in comparison to those using monochloramine [140]. It has been suggested that monochloramine is more effective in penetrating the biofilm than chlorine [141,142]. However, monochloramine showed similar issues to chlorine; while it was able to suppress *L. pneumophila*, it proved incapable of eradicating the bacteria from the system, and ineffective eradication could lead to monochloramine resistance [143–145]. It is currently unknown if monochloramine induces genetic mutations in *L. pneumophila*.

Bromine and iodine have both been effective in disinfecting swimming pools and cooling water. However, bromine is not recommended for potable drinking water, and there is controversy surrounding safe levels of consumption over an extended period with iodine [120,146]. While both are oxidizing agents similar to chlorine, neither is as effective as chlorine at inactivating *L. pneumophila*. Iodine was particularly inept at killing *L. pneumophila* within a biofilm [108,147,148]. Currently, there is no information available as to whether or not bromine or iodine cause genetic mutations within *L. pneumophila*.

Ozone can effectively inactivate bacteria by damaging DNA in both the gaseous and dissolved states [149,150]. Ozone is more effective than chlorine is controlling *L. pneumophila*; however, due to its quick dissipation and inability to be used as residual treatment, another form a treatment is required in conjunction with ozone to treat bacteria within biofilms in premise plumbing [151,152]. Ozone is known to damage DNA, but it is unclear if it causes specific and consistent knockdowns or other mutations in *L. pneumophila*.

Temperature may be the most effective means of controlling *L. pneumophila* in premise plumbing systems. It is recommended that recirculating hot water leaves the heater at least 60 °C and remains at 50 °C throughout the system. Hot water should reach the tap in a maximum of 1 min and be a minimum of 50 °C [153]. Maintaining hot water systems over 50 °C throughout the entirety of the premise plumbing is challenging from both an energy use and a hazard standpoint. Water stagnates for up to 23 h a day within buildings, leading to vast temperature fluctuations [154]. Furthermore, maintaining high water temperatures from the heater to the tap can be costly, particularly if the premise plumbing system is large [154]. Additionally, facilities with large premise plumbing systems are concerned with the risk of scalding, specifically healthcare facilities who may have patients with decreased ambulatory abilities and other health concerns, such as dementia, which may not allow them to recognize the water temperature or move from hot water in a timely manner. Maintaining temperatures high enough to retard the growth of or kill *L. pneumophila* could prove hazardous to the population [155]. Intermittent "super heat" flushing, maintaining the water in the system at 70 °C, and flushing the taps for 30 min has shown to be effective in controlling legionellosis outbreaks [156,157]. However, the logistics of "super heat" flushing may be prohibitive to many facilities.

3.3. Engineered System Stressors within a Biofilm

L. pneumophila is common to biofilms in premise plumbing and is known to have a commensal relationship with protozoa within the biofilms, which can act as protective reservoirs for the bacteria [122,158,159]. As such, it is important that treatments can penetrate the extracellular polymeric substance (EPS) matrix of the biofilm and persist in large premise plumbing systems [160,161]. Chlorine dioxide was shown to be most effective in reducing *L. pneumophila* levels in biofilms in copper piping, compared to chlorine, monochloramine, electro-chlorination, ozone, and copper-silver ionization, due to its longer residual activity and its ability to penetrate the biofilm [162]. However, the amoeba in the biofilm were resistant to all forms of treatment and *L. pneumophila* was able to regrow after short periods of non-treatment [162,163].

High nutrient content, such as iron, organic carbon, nitrogen, and phosphorous support the growth and persistence of *L. pneumophila*, but the bacteria are also resistant against nutrient

depletion [63,126,164,165]. Similar to the bacteria's defense against oxidative stress, *L. pneumophila* can become VBNC when experiencing nutrient depletion [126]. It appears that *L. pneumophila* can survive and thrive in any conventional pipe material. However, the bacteria are most prolific in cast iron pipes and least productive in copper piping, with plastic piping as the middle ground [40,166]. Piping materials can be taken into consideration for new construction projects, but tend to be a cost-prohibitive remediation or preventative factor in existing structures. It is important to consider the type of water distribution system, location, and use before deciding on treatment or mitigation options.

The laboratory and in-field experiments described above have shown that eradicating *L. pneumophila* within the biofilm is challenging if not unrealistic, and periods of treatment neutralization can lead to legionellosis outbreaks. Therefore, it can be postulated that the prevention of legionellosis should focus on decreasing infectivity of *L. pneumophila* rather than eliminating the bacteria from premise plumbing. Utilizing the lifecycle and genetic information discussed in this review and the following replication and egression reviews to create an effective QMRA model for *L. pneumophila* within the drinking water system is a first step in targeting the reduction of the bacteria's virulence and preventing legionellosis outbreaks.

3.4. The Effects of Genetic Knockdowns

Genetic knockdown information, which affects *L. pneumophila's* ability to invade the host cell, was gleaned from the literature (Table 1). Data is only reported in this article if it is sufficient to use in a mechanistic uptake model, requiring that it: was reported in a peer-reviewed journal, had greater than three data points, was validated, had sufficient variation in the independent variable, applied to genes that are responsible for pathogenesis, and was comparable to the other data provided. There is a lack of macrophage studies with appropriate data available in the literature, due in large part to murine studies being inappropriate for this model. Data available in the literature are difficult to use in one comprehensive model as the authors utilize different methodologies, various timeframes and report the effectiveness of the genetic knockdown in different units (i.e., percentage reduction vs. log reduction). In many studies, the knockdown causes only a minor reduction, making log reduction a less intuitive form of communication. In this article, the units expressed in the original paper are reported as well as the degradation of uptake rate, the percentage of bacteria, which were incapable of invading the host cell after the knockdown as compared to the wild-type. It is recommended that future research reports its results in percentage reductions for modeling purposes.

Table 1. Effects of Genetic Knockdowns on the uptake of *L. pneumophila* into a phagocyte.

Gene	Host Cell	Change in Uptake Efficiency *	Degradation of Uptake Rate **	Process
relA	*A. castellanii*	30–40%	30–40% ***	The flagella subunit is not produced, causing reduced anchoring to the host cell [167]
pilE	U937 cells	33.9%	66.1%	Only the short pili is expressed in mutant [80]
pilE	HeLa cells	34.0%	66.0%	Only the short pili is expressed in mutant [80]
pilE	WI-26 VA4	48.7%	51.3%	Only the short pili is expressed in mutant [80]
pilE	*A. polyphaga*	53.4%	46.4%	Only the short pili is expressed in mutant [80]
lqsA	*A. castellanii*	20–35%	20–35%	Diffusible signaling molecule (LA-1) is not produced during mutation [168]
lqsS	*A. castellanii*	40–65%	40–65%	Sensor kinase which recognizes LA-1 is not produced during mutation [168]
letA	*A. castellanii*	Log 3 CFU/L	99.9%	The flagella is not produced in mutant [169]
Mip	NCI-H292 lung epithelial cells	Log 2 CFU/L	99%	Mutant loses the inability to cross the extracellular matrix [170]

* Expressed in units reported in the original literature. ** Degradation of Uptake Rate refers to the percentage of bacteria that were unsuccessful in invading the host cell as compared to the wild-type. *** Refers to the percentage of cells immotile after the *relA* knockdown.

3.5. Postulated Modeling Framework

While an operational modeling framework will require additional research to develop from the data and knowledge in this review, a preliminary one can be postulated. It is clear that the phagocytosis process is vital to appropriately modeling the lifecycle of *L. pneumophila*, and if an accurate prediction of the concentration is desired. Therefore, the phagocytosis process needs to be modeled as well as general *L. pneumophila* growth and persistence in biofilms. It is also clear that the genes that regulate protein secretion and phylogenic characteristics required for effective uptake drive the uptake into host cells. Since the self-induced uptake of *L. pneumophila* is a vital component of its lifecycle, the effects on these genes from environmental stressors must be modeled. Due to the uncertainty and variability of the effects from environmental stressors, as well as the lack of data conducive to modeling, these processes would be modeled best in a stochastic method. Stochastic methods will allow for the uncertainties and variabilities to be accounted for and used in the model estimates. A postulated modeling framework for uptake into the host cell, including human macrophages can be seen in Figure 1. The foundation of environmental quality impacts genetic knockdown, which, in turn, affects phylogenetic outcomes and results in uptake rate degradation. Therefore, the framework in Figure 1 will allow for a mechanistic model of uptake rates due to environmental stress.

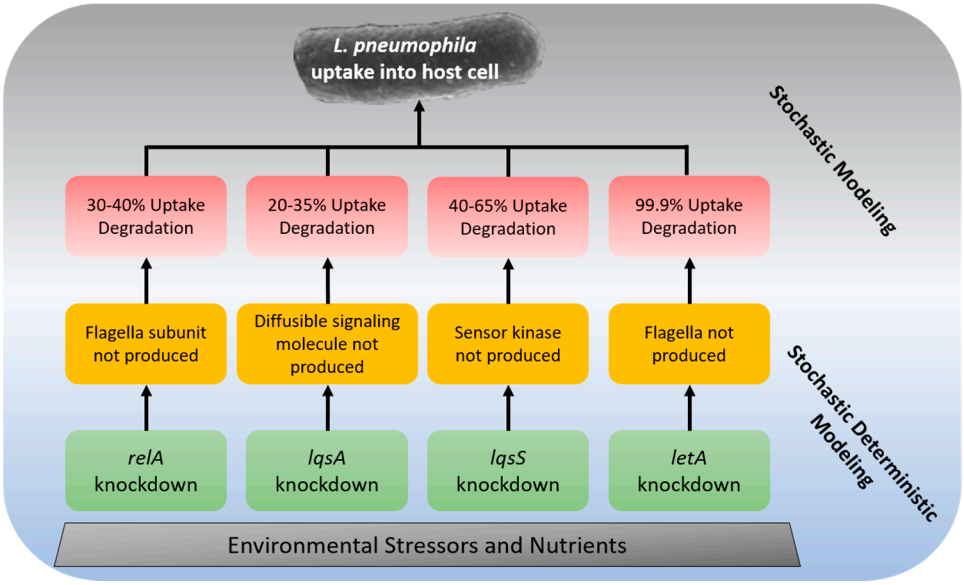

Figure 1. Postulated framework to computationally model the uptake of *L. pneumophila* into host cells.

4. Discussion

Legionellosis is a serious threat to human health and has been increasing in incidence throughout the US [14,15]. *L. pneumophila* thrive in warm, freshwater habitats, particularly where water is stagnant [140,171]. The pathogen prospers where industrial or residential hot water is kept under 50 °C [59,172]. For these reasons, the premise plumbing of hospitals and hotels tend to be natural environments for the bacteria to live and proliferate. Unfortunately, the elderly and immunocompromised are at the highest risk for contracting LD, meaning *L. pneumophila* thriving within a hospital setting put the most vulnerable populations at higher risk. Furthermore, patients with ambulatory impediments may take longer to shower, causing a higher exposure time if *L. pneumophila* is present. Considering the rise in legionellosis incidence, numerous attempts have been made to eradicate *L. pneumophila* from water systems, in particular from biofilms of premise plumbing where the bacteria is significantly more difficult to treat than free living *L. pneumophila*. Prevention of the disease is difficult due to the endosymbiotic relationship *L. pneumophila* has with protozoa within the biofilm of premise plumbing systems [60].

L. pneumophila are particularly resistant to typical disinfection measures due to the ability to use protozoa as a reservoir [122]. There is a good deal of information regarding *L. pneumophila*'s lifecycle and factors affecting the bacteria's virulence and persistence. However, a comprehensive literature review providing information necessary to model the behavior of *L. pneumophila* within a biofilm was not available, necessitating this article.

This literature review focuses on the uptake portion of the bacteria's lifecycle. While the role of many genes and gene complexes have been identified in the lifecycle of *L. pneumophila*, few studies have determined how environmental stressors (e.g., disinfectants), affects these genes [50]. Considering the prevalence of *L. pneumophila* in premise plumbing systems and the difficulty in eradicating the bacteria, reducing the infectivity of *L. pneumophila* may be a more productive solution than vain attempts of killing the bacteria. The most effective treatments of *L. pneumophila* in the premise plumbing system can be logistically challenging, if not prohibitive and costly. Additionally, repeated hyperchlorination events can have a negative impact on the plumbing system, which can lead to infrastructure concerns, other contaminants from the biofilm, and disinfectant byproducts [143]. Times of disinfectant neutralization have been shown to aid in the survival of the bacteria in the biofilm and decrease susceptibility to a disinfectant in the future [137]. Furthermore, *L. pneumophila* occupy a niche in the biofilm. Successfully eradicating the bacteria may allow for another pathogen to occupy the now vulnerable niche.

The mechanisms between *L. pneumophila's* entry into a protozoa and a human macrophage are similar, and the genetic knockdowns which prevent the bacteria from uptake into a protozoan often prevent invasion of a macrophage as well [80,168]. However, *L. pneumophila* has redundancies in the uptake pathway, which allows the bacteria more versatility [77,84]. *L. pneumophila* utilizes multiple pathways to invade host cells, including traditional phagocytosis, coiling phagocytosis and pinocytosis, and multiple receptor-mediated pathways [81–83].

Not only will *L. pneumophila* utilize different methods to infect different species of host, in many cases, the bacteria are capable of utilizing more than one method of invasion on the same host cell. Furthermore, *L. pneumophila* is unique in its ability to attract host cells and initiate invasion as part of its lifecycle [173]. Preventing *L. pneumophila* from entering any protozoa within the biofilm will prove difficult as a result, but doing so will also decrease, if not eliminate, *L. pneumophila*'s virulence. Determining if genetic knockdowns that will decrease infectivity in *L. pneumophila* will occur during the water treatment process is a first step in controlling legionellosis outbreaks. The knowledge and data in this review provide a clearer picture of how disinfection strategies can govern uptake into a host cell. Reduced uptake may be a part of a new means in controlling infectivity. The first step is modeling these processes for which these data are a vital initial foray into this knowledge space [174–177].

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