Molecular Environmental Epidemiology assessment of *Legionella* spp. and *Legionella pneumophila* at the Pineios river.

Department: Ichthyology and Aquatic Environment

Master Programme: HosMic-‘’Host Microbiome Interactions’’

Year: 2024

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Abstract

Legionella pneumophila is a facultative endosymbiotic bacterium and the first bacterium described that multiplied within protozoan hosts, primarily aquatic amoebae that is also an opportunistic human pathogen. It causes a respiratory syndrome with various level of severity by infecting human lung macrophages. Currently, it is a well-known fact that that *Legionella* are found in the environment in parasitic forms, meaning inside their amoebae hosts and ''free-living'' in biofilms. Human infection most commonly occurs as a consequence of inhaling *Legionella*-containing aerosols generated by contaminated manmade water sources.

In our study we attempted to: develop a real-time qPCR based monitoring protocol of genes related to the bacterial species *Legionella pneumophila* and in general of *Legionella* spp. in aquatic environments (from sampling to cell concentration to gene quantification), and assess the effect of temperature in the abundance of these taxa in environmental populations of Pineios river. For achieving our goals we used primers targeting the 16S rRNA (coding a part of the large ribosomal subunit of ribosomes), the 5S rRNA (a ribosomal molecule associated with the large ribosomal subunit) and *mip* (macrophage infectivity potentiator, which codes the main factor of pathogenicity of *L. pneumophila*) as marker genes. For the development of the qPCR methods for our markers, we transformed bacteria with vectors containing PCR products of the tested primer-sets which were used for setting up standard curves for the qPCR-based quantification. Isolation of the recombinant plasmids and insert sequencing validated the methods for LEG (amplifying the partial 16S gene of *Legionella spp.*), L5S (amplifying the 5S rRNA gene of the *L. pneumophila)* and *mip* (amplifying genes that code for the macrophage infectivity potentiator of *L. pneumophila*), according to the comparison with NCBI's nucleic acid databases. Throughout an increase of the ambient temperature of the Pineios river from 29-34˚C, qPCR tests of our water samples showed an increase of the total bacterial 16S rRNA gene, the LEG (16S rRNA gene of *Legionella* spp), and the L5S (Legionella spp specific 5S rRNA gene) markers along temperature increase and time progression. On the other hand, the *mip* marker showed no correlation with the temperature in the temperature range during the surveying period. During this temporal progression and temperature increase we further observed an increase in α-diversity and taxa like *Acinetobacter*, *Chloroflexi*, *Bacilli* and *Pseudomonads*. Overall, we were successful in developing and implementing qPCR approaches for monitoring the legionella targets, and our Pineios monitoring results suggest that temperature plays a significant role in the growth and raise of the populations of *Legionella* spp, yet, the temperature non-correlated pattern of the *mip* marker suggests that *L. pneumophila* has different optima compared with the rest *Legionella* spp in environmental samples that may prevent it from proliferation. Nevertheless, further studies are necessary in order to understand the underlying ecological mechanisms of *Legionella* spp and *L. pneumophila* distributions at the Pineios river.

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Acknowledgements

Firstly, I would sincerely thank Assistant Professor Dr. Vasileiadis Sotirios for the appointment of this thesis and his supervision during the performance of this project. I would, also, like to thank him for his valuable aid and guidance during the organisation and set up of the experiments, interpretation of the results, dealing with any problems and obstacles that occurred during the experiments, as well helping with the interpretation of the results and correcting this thesis. A wholehearted acknowledgment to Assistant Professor Dr. Papadopoulou Evangelia for her participation in this committee. In addition, I would like to express my gratitude to Professor Dr. Karpouzas Dimitrios for accepting me to his group and laboratory and being a member of this committee. A big thanks to my lab partner, undergraduate student Ioannis Koutals for his great aid and collaboration throughout the experiments. I wish him best of luck with his future endeavours. I would also like to thank all the members of the laboratory for their suggestions and advices as well as the great work environment they offered during my thesis. Finally, and most importantly, I would like to thank my family for their unconditional support throughout all my endeavours over the past years. I will always be grateful to them.

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1. Introduction

1.1. *Legionella* in the environment and its epidemiology

1.1.1 *Legionella*

First *Legionella* incidence verified records were provided in 1977 by the Center for Disease Control and Prevention (CDC) after a local outbreak of pneumonia during a convention of veterans of the American Legion that took place a year before in Philadelphia, PA, USA (Mekkour et al., 2013). The veteran's association named the bacterium. (Brenner, 1979). Over 50 species of *Legionella* have been identified to this date including more than 70 serogroups (Mekkour et al., 2013).

Table 1. I Phylogenetic Taxonomy (Brenner et al. 1979)

Taxonomy of *Legionella*	
Domain	Bacteria
Phylum	Pseudomonadota
Class	Gammaproteobacteria
Order	Legionellales
Family	Legionellaceae
Species	Legionella

Legionella spp. has a rod-like shape (bacillus). They do not form spores or capsules. Their dimensions are mainly 0.3-0.9 μm in width and 0.2-2 μm in length. Sometimes, they form coccobacilli. It is documented that this shape is mainly associated with the stage of the culture that the bacteria exist, with a higher frequency in cultures that are more mature. Some species are mobile while others are not and are just adhering to surfaces waiting for a host to come by, due to the current in aquatic environments. The bacteria are mobile with the use of polar or lateral flagella (single or multiple) and cilia. Temperature determines whether mechanisms of mobility exist or not. It is documented that with the rise of temperature, a reduction of the mobile bacteria in the whole population is occurring (Ott et al. 1991).

Legionella bacteria are characterised by omnipresence in aquatic or solid environments, as they can be found in all natural soil and most types of water systems, either natural or engineered (Gonçalves et al., 2021).

Certain strains of *Legionellae*, characterised as Legionella-like amoebal pathogens (LLAPs), are not able to grow in Legionella media, like e.g. buffered charcoal yeast extract (BCYE) (Fields et al., 2002). LLAP strains were identified with the application of DNA homology methods, especially with the genotyping using the *mip* gene (Benson et al., 1996; Ratcliff et al., 1998) in the mucus of pneumonia

patients when they were cultured along with protists. It is clear, that human *Legioenella* pathogens are also part of this group of microorganism (Bartram & Weltgesundheitsorganisation, 2007). It is difficult to prove that all LLAP *strains* are human pathogens, mainly because not all the strains of LLAP's which were cocultivated with protists and patient's mucus, were found in the cells of the patients. The most plausible explanation is that inhalation of infected protists with LLAP's was occurred and thus the LLAP's cannot infect human cells in their free-living form. That could also explain why they are not detectable using standardised routine bacteriological methods used for *Legionallae*. (Adeleke et al., 2001; Maltezou et al., 2004).

The representative species of *Legionella* and the subject of this thesis, is *L. pneumophila*, one of the first species of the genus to be described and of clinical interest, since it is an opportunistic pathogen and a causal agent of legionellosis. This condition can be severe, with all pneumonia symptoms (usually in older, immunosuppressed or people with respiratory disease records) or mild, usually in younger people called ''Pontiac fever'' (Rowbotham, 1980a).

1.1.2 *Legionella pneumophila*

1.1.2.1. Legionella pneumophila physiology and needs for cultivation

Legionella pneumophila is a small, Gram$^-$, aerobic bacterium that doesn't form capsules or spores (as almost all the strains of the *Legionellaceae* family), that possesses the enzymes of catalase and oxidase. The genetic material is constituted between 38 and 52 % guanine and cytosine (Brady & Sundareshan, 2023). The bacterium is approximately 2 μm in length and 0.3-0.9 μm in width. *L. pneumophila* is a facultative intracellular parasite. It is not known to multiply in clean water, but grows very well when cocultivated amoeba cultures that are swimming freely (Breiman et al., 1990). *Legionella pneumophila* processes 2 polar flagella when is motile, with its motility being temperature-dependent at temperatures ranging between 30 °C and 40 °C (Ott et al., 1991).

L. pneumophila is a fastidious organism and will not grow anaerobically on a standard media. Buffered charcoal yeast extract agar (BCYE Agar) is the medium commonly used for its isolation (Brady & Sundareshan, 2023). *L. pneumophila*'s auxotrophy is mainly documented for various aminoacids. The bacteria are fastidious for the amino acids L-arginine, L-isoleucine, L-leucine, L-methionine, L-serine, L-threonine, and L-valine, as well as, for L-cysteine (or L-cystine) in some cases (Tesh & Miller, 1981).

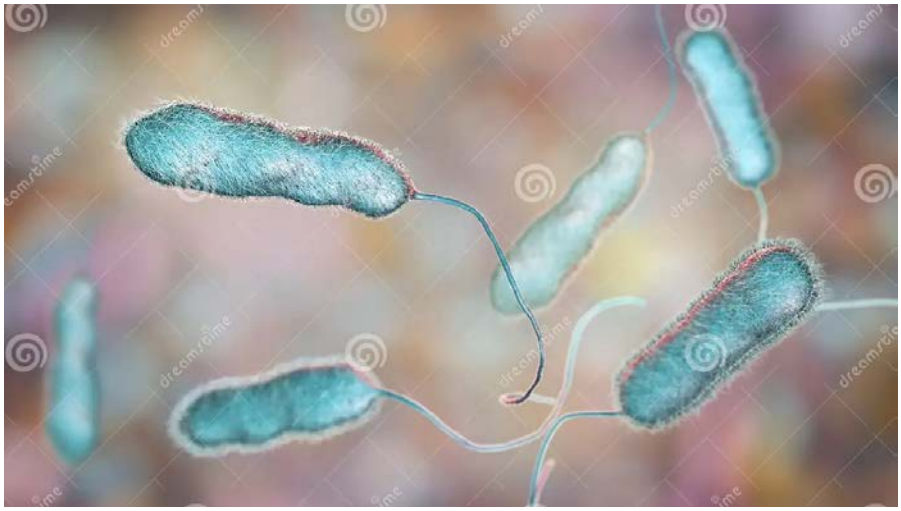


Figure 1. *Image of Legionella pneumophilla bacteria.*

1.1.2.2. Legionella pneumophila virulence

L. pneumophila can multiply intracellularly in human macrophages (Horwitz & Silverstein, 1981). The processes by which legionella infects protozoa and phagocytic cells of mammals are extremely similar (Garduño et al., 2002; Horwitz & Maxfield, 1984). *Legionellae* have a comparable life-cycle within protozoa, which are their natural parasitic targets and human macrophages. *L. pneumophila*, which contains the relevant virulence factors, may infect and replicate in different protozoa found in soil and water. By doing so, it can increase its virulence (N. Cianciotto, 2001).

In vitro liquid culture studies demonstrated that the life cycle of *L. pneumophila* consists of at least two discrete phases: i) in their free-living phase, the bacteria are highly motile, virulent (able to evade from host cell's defences) and flagellated; ii) in their replicative phase, taking place in the replicative vacuole, they are unflagellated and resistant (Molofsky & Swanson, 2004; Swanson & Fernandez-Moreira, 2002). These two phases also exist in vivo during the infection of amoebae. This can be confirmed using intracellular gene expression profiles of *L. pneumophila* (Brüggemann et al., 2006). However, under certain conditions, intracellular *L. pneumophila* may differentiate to a spore-like form (Garduño et al., 2002), while *L. pneumophila* may also reside in biofilms (Piao et al., 2006). Thus, the *L. pneumophila*'s biological cycle could be more complex depending on the conditions under which it is studied. Regulation of the shift from the replicative to the transmissive phase in L. pneumophila's requires a complex regulatory network, which is only partially understood. (Fragou et al., 2012).

Identification of genes enabling an organism to infect both protozoan and human cells was studied for the *L. pneumophila* and was associated with its pathogenic potential, yet, the postulated underlying mechanism was not associated with all *Legionella* species (Ogawa et al., 2001).

1.1.2.3. Legionella pneumophila pathogenicity mechanisms

Legionella blocks phagocytic bactericidal activity and makes the phagosome a niche for replication (Fields et al., 2002; Yu & Stout, 2000). In this compartment that resembles the host endoplasmic reticulum (ER) L. pneumophila establishes a replicative niche by controlling the membrane traffic and fusion machinery of the host. This process is carried out by bacterial proteins named **Legionella effectors** that are transported to the host cytosol. Legionella bacteria produce ER-derived vesicles (ERDVs) in the early time period after the host infection. These vesicles are used to create the

Legionella-containing vacuoles (LCVs). Furthermore, the LCVs associate with the ER in a mechanism that remains elusive (Kawabata et al., 2021).

Aside from the molecular mechanisms previously mentioned, post-translational modifications (PTMs) are essential for controlling the biological responses of cell to environmental changes. *L. pneumophila* recruits a variety of post-translational and post-transcriptional modification mechanisms to increase its pathogenicity. Hijacking of host cell vesicle trafficking allows for LCV biogenesis, and subsequently for intracellular multiplication *of L. pneumophila* cells. An essential component of the cell cycle of the bacterium. LCV biogenesis is performed through the type IV secretion system T4SS (Andrews et al., 1998), which translocates over 275 bacterial proteins/effectors in the cytosol of the host cell (Zhu et al., 2011). Many of these effectors are associated with specific domains, such as protein–protein interaction domains and enzymatic activity-associated domains, whereas performed post translational modifications including methylation, phosphorylation, ubiquitination, and glycosylation. Associated studies provide evidence in favour of the theory that *L.pneumophila* encompasses post-transcriptional modifications mechanisms resembling those of eukaryotes for facilitating the hijacking of host cells via interference with the native cell machinery (Cazalet et al., 2004).

1.1.2.4. L. pneumophila's MIP protein

Surface structures play an important role in the pathogenicity of *Legionella* (N. Cianciotto, 2001; Heuner & Steinert, 2003). Along with the flagellum and the pili, certain bacterial surface proteins are involved in the adherence and entry of *Legionella* into alveolar macrophages and protozoa. These proteins include: (a) the major outer membrane protein (MOMP), (b) the heat shock protein (Hsp60) and (c) the macrophage infectivity potentiator protein (MIP) (Bartram & Weltgesundheitsorganisation, 2007).

The effector that is clearly linked to viral capabilities of the bacteria is the 24-kDa macrophage infectivity potentiator (MIP) protein, coded by the *mip* gene (Fields, 1996). The MIP protein is believed to be conserved across the genus (Ratcliff et al., 1998). It is required for the infection of both mammalian phagocytic cells and protozoa (N. P. Cianciotto & Fields, 1992). However, in the case of intracellular infections, other effectors (such as proteins and nucleic acids) are crucial along with the activity of the MIP protein (Karagöz et al., 2022). An example is that AnkB (an F-box eukaryote like) protein interacts with the host's cellular proteins. Once inside the host cell, AnkB protein suppresses the host's immune response by inhibiting the production of reactive oxygen species (ROS) (Bruckert & Abu Kwaik, 2016).

MIP is suspected to have been involved in the infection, collagen binding, phospholipase C activity, translocation of tissue barriers, Nematocyst colonization, surface translocation and growth at a low temperature (Scheithauer et al., 2023).

MIP is homologous to eukaryotic FK506-binding proteins (FKBPs) as far as amino acid sequences are concerned. FKBPs belong to the substance class of the immunophilins. These proteins, classified as peptidyl-propyl cis/trans isomerases (PPIases), act as catalysts of various isomerisation of prolyl bonds in oligopeptides and proteins. In addition, immunophilins are crucial in regulating T-cell signal transduction pathways (Ludwig et al., 1994). The adjustment of transcription patterns, allow the host's immune system, through phagocytosis and chemotaxis of T-cells, to eliminate the invading intracellular parasitic bacteria of Legionella. The interaction of MIP with lncRNAs and other non-

coding RNAs has also been demonstrated. A series of posttranscriptional regulations of genes, modify the expression of non-coding RNAs. These RNA molecules are critical for post-transcriptional control of the mediation of the phagocytosis of the pathogens in the macrophages. With these interactions MIP interrupts the normal functions of cell mediated immunity of the host (Shen et al., 2022).

1.1.2.5. Adjunct factors of L. pneumophila's pathogenicity

Factors enhancing the *L. pneumophila* extracellular survival and virulence capacity are the extracellular vesicles (EVs) formed after host infection EVs of *L. pneumophila* contain a plethora of immunomodulatory and surface-associated proteins and lipopolysaccharides (LPS) on the outside. They act as pathways of communication for the bacteria, but it also possible that they are associated with host cells and can transport a plethora virulence factors. One of these effectors is MIP. Another type of virulence factors are RNA molecules. It has been addressed that RNA molecules produced by *L. pneumophila* mediate pathways of the host's for recruitment of interlefkins, aiding to it's infectious ability (Newton et al., 2010). The LPS can act as an adhesion factor that anchors the bacterium in the host cell membrane (Scheithauer et al., 2023).

Other virulence factors concern *Legionella* motility A sigma factor σ^{28} (*FliA*) is responsible for the initiation and the regulation of the transcription of *fllaA* (flagellin gene), that encodes for flagellin (the main molecule of the flagella of *L. pneumophila*) (Heuner et al., 1995). Expression of gene and motility are primarily determined by the growth phase, but also by environmental factors such as the availability of amino acids and the osmolarity of the medium (Heuner et al., 1999). On the functional level, it is demonstrated that MIP promotes *L. pneumophila* flagellation.

MIP or FlaA through their in-between interactions and with other factors, can mediate and regulate the infection ability of *L. pneumophila* (Scheithauer et al., 2023). For the *L. pneumophila* environmental fitness and pathogenicity, control of the flagellation, mediated by FlaA and the extracellular release of the bacterium, is important. The pathogenicity of the bacterium and motility is further demonstrated to be strongly influenced by a tripartite interaction between 3 genes: *mnp2*, *flaA2* and *lpc2601* (responsible for lipopolysaccharide exudation) (Karagöz et al., 2022).

Mechanisms of host manipulation for the survival and proliferation of *L. pneumophila* further involve small RNAs. it is known that small RNAs of *L.pneumophila* mimic eukaryotic microRNA and modify the host's response to infection making the host increasingly susceptible and enabling *L. pneumophila* to survive and proliferate (Sahr et al., 2022).

1.1.2.6. Survival mechanisms of L. pneumophila

In order to withstand adverse conditions, such as scarce nutrients, temperature extremes and the effects of biocides, *L. pneumophila* creates biofilms. Polysaccharides are secreted by the cells and gradually form a material that aids in the surface adhesion of the bacteria. The secreted polysaccharides form a polysaccharide matrix that the cells attach. In the case of *Legionella*, a small colony of bacteria, along with some protists, attaches to the surface. Then these cells secrete 4-hydroxy-5-methyl-3(2H)-furanone (HMF) molecules. This allows communication with other Legionellae bacteria and their recruitment through quorum sensing. The secretion of more ingredients from bacteria will lead to the accumulation of the hosts and bacterial cells and slowly as more bacteria, hosts and ingredients are gathered, until a new environmental niche is created, that

of the biofilm matrix. It has been established that biofilms are the most favourable niches of ''free living'' Legionella bacteria (Abu Khweek & Amer, 2018; Armon et al., 1990). Except from the formation and maintenance of biofilms, quorum sensing regulates flagellar synthesis, motility, and the production of virulence factors (Abu Khweek & Amer, 2018). In nature, biofilms are formed on rocks or other solid surfaces in water bodies, but in engineered systems, they are most prominent on galvanized steel surfaces. The development of biofilms is influenced by a number of parameters, these are mainly the following: the lack of system maintenance, the high consistency of the water with salts, stagnation of water flow, the chemical consistency of the water and the materials that the pipelines of the system are constructed from (Fragou et al., 2012).

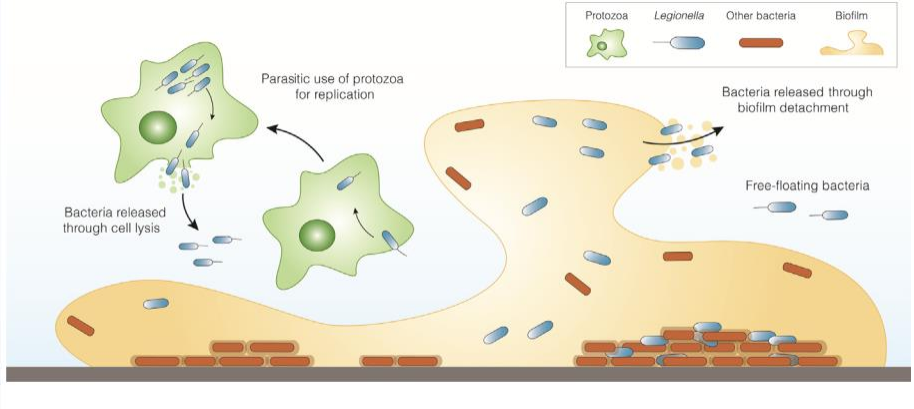


Figure 2. Formation of biofilm by Legionella pneumophilla bacteria (Gonçalves et al., 2021).

1.1.3 *Legionella* in aquatic environments

Legionella was the first bacterial taxon known to reproduce within protozoa/amoebae. Protozoans have many similarities with the human macrophage cells and thus it was inferred that the bacteria were able to reproduce within human lung macrophages (Rowbotham, 1980a). That was later confirmed through a number of studies (Brady & Sundareshan, 2023) and it is currently, acknowledges as a fact that that *Legionella* exist in the environment as parasites, inside their amoeba hosts, or as ‘’free-living’’ in biofilms (Gonçalves et al., 2021).

Legionellae, both in natural and man engineered water systems are able to survive in a wide range of conditions (Fliermans et al., 1981). They have been isolated from environmental samples of pH ranging from 2.8 to 8.3 (Anand et al., 1983; Sheehan et al., 2005). In water springs and subterranean waters, as well as in salt water or near a mountain spring, *Legionellae* have been detected (Brooks et al., 2004; Campbell et al., 1984; Riffard et al., 2001).

One of the key factors for *L. pneumophila*’s survival and growth, is temperature. The bacteria are isolated from water systems at a temperature of of temperatures of 20 ºC to 70 ºC (Fliermans et al., 1981) with temperatures above to 70 ºC being inhibitory for their survival (Dennis et al., 1984). Also it has been proven that 48.5 °C-50 °C is the highest temperature the bacteria can grow (Kusnetsov et al., 1996).

Optimal *Legionella* growth temperatures range of inbetween 32 ºC and 42 ºC, while *L. pneumophila* can survive and multiply between 25 °C to 45 °C (Yee & Wadowsky, 1982). The most common temperature of isolation of the bacteria is between 37 °C and 42 °C (Schulze-Röbbecke et al., 1990; Yee & Wadowsky, 1982). The growth rates of bacteria are very low when temperature drops below 37 °C, while in the 20 °C temperature level there is almost no growth. Under these low

temperatures, bacteria in the population tend to remain dormant for a period of time and grow again if conditions become favourable (Yu & Stout, 2000).

L. pneumophila has been repeatedly shown to be facultative endocellular parasites at *in vitro* studies. They have proven their ability to multiply in cells of different amoebal species and cilliophorale protists (*Tetrahymena*) (Fields, 1996; Fields et al., 1984; Hägele et al., 2000; Rowbotham, 1980b). In all water systems, both natural and artificial, protists serve as hosts and carriers, greatly affecting *Legionella*'s survival capacity to survive under various environmental conditions. Since *Legionella pneumophila* cannot infect all protists, it is safe to assume that there is some degree of host-specificity between the two components of the parasitic relationship (Quinn & Tompkins, 1989).

From the moment the bacteria are introduced into the host, the have the ability to remain there almost indefinitely, until the environment becomes favorable for their growth and prolifration. The bacteria can only multiply at 35 °C, but they can enter in their hosts at temperatures as low as 22 °C. (Nagington & Smith, 1980; Rowbotham, 1980b). It has also been shown that in the cases of protists and monocytes, *Legionelae* with cillia have a much higher efficiency in parasitism compared with those without it (Heuner & Steinert, 2003).

Among the factors influencing the survival and growth of *L. pneumophila*, most important are those of temperature and availability of hosts and nutrients. The population of *L. pneumophila* in the environment rises in analogy with the sediment content. This is because the sediments contain a number of substrates that can serve as growth for *L. pneumophila* cells (Stout et al., 1985). Another factor affecting *L. pneumophila* growth is that of concentrations of various metals and salts. Mainly, those consisting of cations, such as magnesium and calcium (Vickers et al., 1987). It is also hypothesised that small concentrations of iron, zinc and vanadium could lead to the proliferation of *Legionella* (Stout et al., 1992).On the other hand, high concentrations of copper, iron, manganese and zinc inhibit the growth of *Legionella* (Kusnetsov et al., 1996). Furthermore, it is demonstrated that the proliferation of bacteria in sterilised water is not feasible. They can endure for extended periods of time in the sterilised water but are dependent on either the existence if their hosts organism or direct sources of nutrients from the environment for their proliferation (Fields et al., 1984; Stout et al., 1985; Yee & Wadowsky, 1982).

Inhalation of *Legionella* carrying aerosols from water sources such as hot tubs, air conditioners, plumbing networks and showers is the dominant mode way of Legionnaires' disease dissemination. Human-to-human is not considered an important transmission method. Only a single case has been reported, raising concerns that it does indeed occur, albeit extremely rare. In general, bacterial infection of humans is coincidental and a dead end for bacteria. At higher risk of infection by Legionnaires' disease, lie people with comorbidities such as smokers and people with underlying medical conditions such cancer, immunosuppression and diabetes, as well as smoking. Also age and sex seems to play a role as well, as male individuals older than 50 years of age have elevated risk of Legionaries' disease contraction and severe illness. Nonetheless, the disease can strike anybody. The majority of people reported of being infected with Legionnaire's disease occur in the summer and early autumn. This time of year's frequent temperature variations combined with elevated frequency of rainfall may be the reason for this (Mondino et al., 2020).

1.2 Diagnostic methods of *Legionella* strains in environmental samples

1.2.1 Current culture-based and culture free approaches (advantages and disadvantages)

L. pneumophila is detected today with numerous techniques for both culture-based and culture-free detection of the microbe. The isolation of an organism or a strain for research comes along with pros and cons, as explained below culture-free approaches are employed in conjunction with culture-based approaches to address culture-based analysis issues. (Toplitsch et al., 2021).

1.2.1.1. Culture-based

The ''culture based'' methods are employed for the cultivation of *Legionella* in specific media to cover the needs of the fastidious lifestyle. One advantage of the isolation of microorganisms is that only living bacteria are being analyzed. Also, taxon specific media provide very high sensitivity for the specific taxonomic group (Figdor & Gulabivala, 2008). Microbial isolation can result in the in-depth genetic, phenotypic, physiological, metabolic, and biochemical characterization of the isolate at hand.

Nevertheless, microbial isolation is achievable only for a small portion of known microbial diversity, while axenic cultures may only provide information about the fundamental niche (set of abiotic/biotic resources an organism can exploit) of the isolates, rather than the real niche (aka, the set of abiotic/biotic resources an organism actually exploits). Strains are auxotrophic in nature for an array of nutrients and growth factors thus, it is difficult to generate appropriate media that replicate their actual environment and cover their needs, particularly without any prior knowledge (Stewart, 2012). Another disadvantage is that these methods fail to capture the quantitative, and ecologically meaningful information of microbial growth in their natural habitats. Another shortcoming of culture based methods is that they require time and resources, for example many bacteria including *L. pneumophila*, are known to grow very slowly in media ((Figdor & Gulabivala, 2008; Toplitsch et al., 2021)). Such pitfalls may be resolved through the analysis of the microbial cells and their genetic content *in situ*.

1.2.1.2 Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) is a method that takes advantage the complementarity of oligonucleotide probes with specific regions of DNA or RNA. The technique makes use of probes that are bound to fluorophores, which are excited by photons of particular energy and emit visible light (Gozzetti & Le Beau, 2000)). To fix the cells, standard FISH protocols use paraformaldehyde for the cross-linking of the cell components, which are further exposed to perforating agents like lysozyme that attacks peptidoglycans, thus making cells more accessible to probes. Following, suitable denaturants are being used for the denaturation of nucleic acids and the exposure of probe targets. Once inside the cells, the probes can hybridize with their targets.(Batani et al., 2019). It is worth mentioning that the probes cannot be used only for DNA-DNA interactions but also for DNA-RNA

interactions. Thus, targeting rRNA nucleic acids (existing in large amounts in the cells) in a FISH setup is a very commmon approach, allowing the presence of multiple probes (and, hence, multiple fluorophores) in a single cell, therefore enhancing the emitted light by the fluorophores per cell and facilitating cell detection. Species-specific probes can be used to identify various organisms in the microbial community (Bottari et al., 2006). Most significantly the implementation of FISH provides important information about the spatial distribution of microorganisms, enabling a thorough understanding of interactions between different species. In the following table the various advantages and disadvantages of this method are shown (Gozzetti & Le Beau, 2000).

Table 2. Advantages and disadvantages of fluorescence in situ hybridization (FISH).

Fluorescence in situ hybridization (FISH)	
Advantages	Disadvantages
Rapid technique and a large number of cells assessed in small periods of time	Restricted analysis for the species that can be detected with currently available probes
Effeciency and specificity of hybridization are high	Only a few species/interactions can be assessed simultaneously
Data can be derived from ''poor'' samples that contain very few cells for routine analysis	Due to failure to detect the signal ,FISH has a threshold of sensitivity due to the use of fluorescence of the probes
Technique permits direct morphological and genetic information about the microorganisms	Processing of samples in general is relatively easy; however the preparation of frozen bacterial samples can be challenging
information of spatial distribution of microorganisms/understanding of inter-species interaction	

1.2.1.3 PCR-based

DNA is isolated either from extracted cells or directly from the analyzed environment and amplified using PCR (Figdor & Gulabivala 2011). The approach relies on the DNA polymerase's ability to add a nucleotide to the 3'-hydroxy group of the deoxyribose via the formation of phosphodiester bonds, resulting in the extension of the nascent DNA strand with polymerisation. The very typical PCR-setup requires the following reagents and components: A DNA template, a DNA polymerase, two complementary DNA primers to target sites, one at each of the opposite strands, deoxyribonucleotide triphosphates (dNTP's), a buffer solution of the proper pH and ionic strength and bivalent Mg^{+2} cations (acting as co-factors at the polymerase active site, interacting with the negative oxygen charges of the phosphate groups of the DNA-strand backbones). The basic steps of a PCR are three and repeated in each cycle and they are (i) denaturation , (ii) annealing (iii) elongation/polymerisation ((Papanikolaou et al., 2016a). Owing to intrinsic variations in their sequences, some genes or regions of DNA allow us to distinguish between organisms belonging to the same or different species. These regions are termed ''molecular/genetic markers''. The methods take advantage of these regions to identify a specific genome or differences between species. Most

molecular approaches to the characterization of bacteria use ribosomal RNA (rRNA), which is mainly used for the identification and relationships between organisms. The *16S* rRNA gene offers signatures, facilitating identifications for a given phylogenetic group of bacteria or microorganisms. Targets of amplification would be non-conserved regions, while the conserved regions could serve for of the designing of primer sites. Other genes that code for rRNA sequences, like the *23S* rRNA and *5S* rRNA genes, are used additionally as targets (Church et al., 2020).

Aside from rRNA coding genes, other loci of the bacterial genome (-typically protein coding loci-) have been utilized to identify different species. Furthermore, particular genera or species of bacteria generate particular products (e.g. agents of pathogenicity). The genes that are responsible for these products provide the capacity to further specify and differentiate the analysis at various taxonomy levels, such as at species or genus level. (Santos & Ochman, 2004). The method used for the monitoring of the amount of amplification of the DNA-molecules is real time PCR. In this method the fluorescence is measured after each cycle and the intensity of the fluorescent signal is analogous with the quantity of the DNA at that cycle. The initial number of template DNA molecules in the sample determines the point at which the fluorescence intensity passes set threshold, used for comparing the analysed sample with samples of known target DNA content (standards). The cycle number that this occurs is referred to as the quantification cycle (Cq) (Kralik & Ricchi, 2017). Every strategy has pros and cons and the culture independent strategies like qPCR are no different. These are summarized in the following table.

Table 3. Advantages and disadvantages of culture-independent methods (PCR,qPCR.) (Figdor & Gulabivala, 2008)

Culture independent/Molecular approaches (PCR, qPCR)	
Advantages	Disadvantages
Analysis of DNA sequences (depending on the probes and preparation of samples the targets of PCR and qPCR reactions are specific).	Risk of contamination (especially for qPCR).
Ability to extract most of an environmental sample's microbial sequences.	Depends on pre-existing sequences in the databases.
Only simple bench work abilities are necessary.	Relies on well designed and specific primer sets.
Relatively simple and fast procedures.	DNA template is influenced by lysis method due to recovery limitations.
Samples can be stored in freezing temperatures (mostly DNA as the freezing-unfreezing cycle can damage RNA, but it can be done with RNA as well) and after some period be used for the experiments. High specificity.	Inability of differentiation between living and dead cells (apart from RT-PCR and qPCR methods).

1.2.2 Novel study methods of *Legionella*

Novel methods for studying of *L. pneumophila* microorganisms include sequencing based approaches, such as phylogenetic marker gene amplicon sequencing analysis and shotgun metagenomics. The most popular sequencing method is that of analysing microbial populations is 16S ribosomal RNA (rRNA) gene amplicon analysis via next generation sequencing methods. Initially, sample concentration and purity is determined by a combination of methods including Nano Drop, QUBIT or agarose gel electrophoresis. With the use of appropriate primers that target particular regions that a *16S* rRNA (typically the hypervariable regions of V3 or V4) allow the distinguishing between organisms can be achieved with the results of a PCR (Ranjan et al., 2016).

Advantages of the method include: (i) the low cost of the analysis per sample compared with previously employed approaches (e.g. multiplexing has reduced the costs to 15-80€ per sample, or even lower depending on the experience of the lab preparing the libraries); (ii) the high sensitivity and resolution of the method compared to previous methods (e.g. traditional methods like clone sequencing of PCR products and denaturant gradient gel electrophoresis – DGGE – provide the ability to interrogate a few 10s of strains/taxa per sample, whereas NGS throughout can be at least 3 orders of magnitude higher than that); (iii) the throughput of the method is scalable and very high (e.g. DGGE can process 15-25 samples at the time, whereas a MiSeq run can fit ~400 samples); (iv) the method provides directly the sequence information of the PCR products of each sample instead of only a fingerprint (like e.g. in the case of the DGGE); (v) the generated data are compositional (hence there are certain advantages like, e.g. the abundance of each taxon-representative-sequence compared with the rest sequences of the same sample will be highly consistent, irrespectively of how many times the sequencing process is repeated, while in the case of e.g. isolation-based counting, large differences may arise due to operator related biases). Besides the advantages, like it happens with all methods there are certain disadvantages like: (i) it is a PCR-based approach , and, hence PCR associated biases interfere with the result interpretation; (ii) despite the, apparent, low sequencing error rates, enhanced sequencing depths result in magnification of the errors and generation of artificial microbial diversity; (iii) the compositionality trait of the method may be a disadvantage in the case that we need to draw quantitative conclusions (Gloor et al., 2017);(iv) computational biology and bioinformatics skills are required, which are not often available; (v) proper data analysis skills are frequently missing from research laboratories, and as a result performed data analysis methods may lead to mis-interpretation; (vi) it is a relatively new method, hence, the appropriate data analysis strategies per type of dataset have not yet been standardized.

Another approach for accessing the genetic information of environmental microbiomes is that of shotgun sequencing of the environmental DNA. This method is based on the sequencing of fragmented environmental DNA and either (i) mapping of the sequencing reads onto reference databases, or (ii) assembling of the sequence reads into contiguous sequences (contigs) in an attempt to reconstruct the DNA fragments of origin of the reads. Regarding the latter approach, and using a variety of evidence these contigs can be further combined into scaffolds and/or metagenome assembled genomes (MAGs) (Popescu & Cao, 2018)(Ranjan et al., 2016). Major advantages of this approach include: (i) the low number of experimental biases attached to this method (e.g. no PCR bias exists); (ii) the large amount of information provided (e.g. both taxonomic and functional information can be derived). Major pitfalls of the method include: (i) the, still, high costs of shotgun sequencing (e.g. 150-200€ or more per sample); (ii) the relatively low coverage of the environmental

microbial genetic content in the case of complex environments like foe example soil. Such pitfalls make the analysis of legionella difficult, particularly in complex environments given its not necessarily high, relative abundance. It is, hence, imperative to introduce *Legionella* enrichment methods.

One advantage of the method is that requires only one field sample, and the protocols for extracting DNA are very simple. Additionally, samples concentration and purity is determined by using Nano Drop, QUBIT or agarose gel electrophoresis. The next benefit of the method is that the option for the appropriate primers is relatively simple for the amplification of the gene regions (for example hypervariable regions of *16S* and *18S* rRNA genes) of microorganisms of interest. Except from that of massively parallel DNA sequencing on next-generation sequencing platforms and computing data analysis can be performed. (Popescu & Cao, 2018). The limitations of this method is that the annotation is dependent on the taxonomic units (OTUs) that they themselves are based on the association of *16S* rRNA gene sequences. The operational taxonomic unit is a vague concept and frequently has different meaning to different researchers. It has been demonstrated that for analysis at the species level, the OTUs are frequently insufficient. Furthermore, certain genes are predicted with the utilisation of the OTUs rather than being sequenced directly. Due to horizontal gene transfer and the existence of numerous bacterial strains, the understanding of the analysis may be limited. However, since Legionella is not a common plasmid carrier, the failure to identify horizontal transfer elements to the chromosomal elements wouldn’t be an issue in our situation.

1.2.2.1 Cell enrichment as an approach for addressing the pitfalls of study methods

As mentioned above the novel study methods of *L. pneumophila* are characterized by some pitfalls, the most important of them being their inability of detecting the bacteria of interest and/or achieving sufficient levels of coverage due to the existence of the bacteria in low numbers.

The solution to these drawbacks is in several cases the enrichment of *L. pneumophila* cells in the samples. For the enrichment of specific bacterial cell types from the samples, a few non-culture-based approaches can be applied. These include centrifugating, filtration, micromanipulation and the use of optical tweezers.

Centrifugation is relatively simple and suitable even in the field because it doesn't require strict protocols or expensive and elaborate equipment. There is a range of centrifugation techniques available for separating cells. The most fundamental and quickest centrifugation method is differential pelleting (Figure 3). According to method's principles particles are separated by their size. Smaller, lesser dense particles precipitate more slowly than larger, denser ones, so under certain conditions (i.e. a particular g-force and centrifugation timer period). The latter will mostly stay in the supernatant. Differential pelleting results in efficiently separating the cells. For the improvement of the effectiveness of the separation, density gradients could be applied. Depending on the needs of the experimental procedures, two general types of density gradient-based methods can be applied: Firstly, the technique of ''rate-zonal centrifugation'', depending on their size and shape, allows the separation of particles with similar densities. The primary determinants of the particles' precipitation time during their migration along a density gradient are their physiological characteristics (mass, shape, lenght etc,). In comparatively brief centrifugation times with low-speed, particles accumulate in the gradient's tube upper section and in the lower section during longer concentration times. Secondly, ‘’isopycnic density gradient centrifugation’’ facilitates the separation of particles with varying

densities. A long centrifugation period at high velocity is employed. As a result, the target particles gather in the same gradient zone. (Fig. 3) (Hinzke et al., 2018).

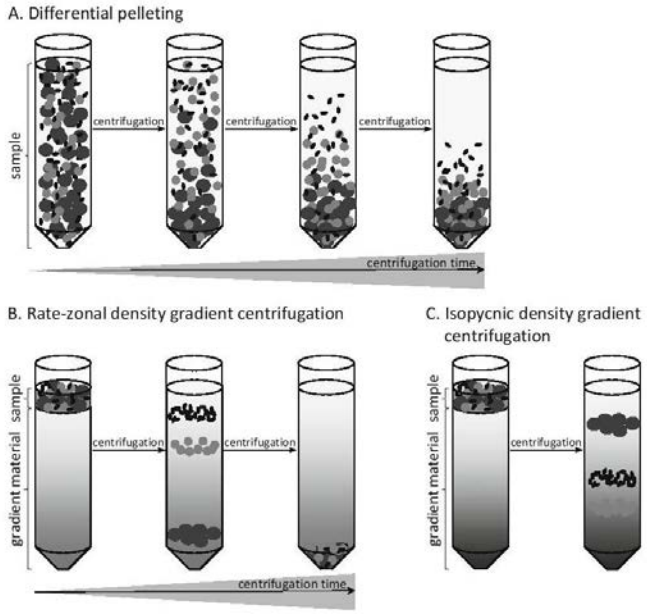


Figure 3. Overview of centrifugation-based cell separation methods. (a) Differential pelleting, (b) Rate-zonal density gradient centrifugation, (c) Isopycnic density gradient centrifugation (Hinzke et al., 2018).

Sample filtration, in which the cells are sorted according to their size, can be beneficial for initial sorting in the primary inoculum if the target group of organisms of interest are particularly small or large in size (Hugenholtz, 2002). Given the high complexity of the environmental samples and the low resolution capacity in terms of morphology-based taxonomic differentiation, such as species and phylum, success not guaranteed using centrifuging as the sole method. Higher concentrations and more pure samples are advantageous for subsequent analyses of each organism of interest, as in the case *L. pneumophila* (Hinzke et al., 2018). For the concentration and treatment of substances according to their physicochemical characteristics, **filtration** is also used. This method is characterised by simplicity and is used in a number of laboratories to remove unwanted particles from the solution and to prepare samples for analysis (*Sample Preparation by Filtration*, n.d.). Additionally, the ''backflash'' filters are ''self-cleaning'', providing the ability to the elements of the filter to lose adhesion from the membrane with the reversal of the flow (J.-H. Kim & Oh, 2019).

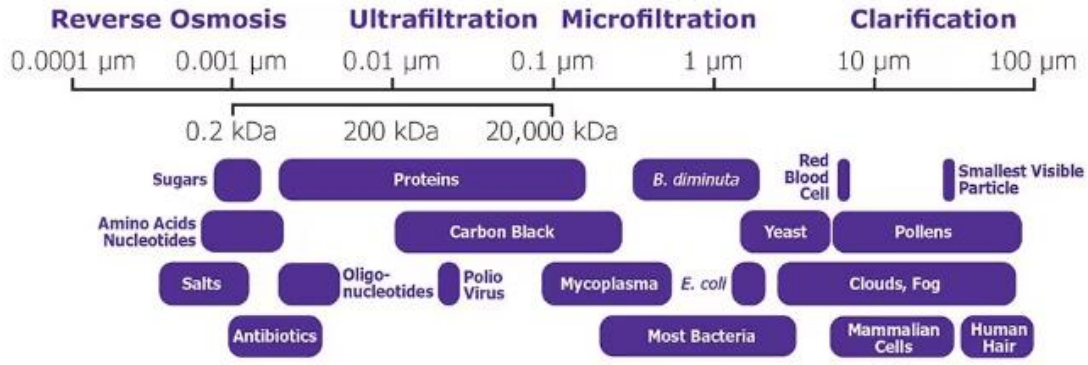


Figure 4. Filtration of various molecules, organisms and particles according to their physicochemical properties (Sample Preparation by Filtration, n.d.).

1.2.2.1.1 Fluorescence in situ hybridization based sorting

One of the most frequently used techniques for cell sorting is fluorescence in situ hybridization (FISH) with ribosomal RNA targeted oligonucleotide probes. It is a quick and a sensitive method and thus a very effective one for studies in ecological and environmental microbiology. A thorough grasp of inter-species interactions can be obtained by using species-specific probes to identify various microorganisms in microbial communities of interest. This knowledge of the composition of microbial communities in natural habitats can be interesting for ecological reasons in microbial ecology, and for safety and technological aspects in food industry, healthcare and primary research (Bottari et al., 2006).

1.2.2.1.2 Immunoprecipitation

Immunoprecipitation (IP) is the method of precipitation of various antigen carrying agents of interest with the use of an antibody that specifically binds to that particular epitope. Bacterial antigens are molecules in the surface of the microorganisms that can interact with antibodies which can, in turn, be attached to moieties that allow their labelling or their further manipulation. A widely used system for immunoprecipitation of the antigen-carying agent (e.g. bacterial cell) is that of biotin-streptavidin interaction, used in a variety of different applications including bacteria cell enrichment (Diamandis & Christopoulos, 1991). The system is based on the interaction of bacterial antigens with a biotinylated antibody, which, in turn, gets attached to streptavidin coated magnetic beads, which can be separated from an aquatic solution with the use of magnets (Marszałł et al., 2008). The method was coined as **Immuno-magnetic separation (IMS)** and has been developed some time ago (Wang et al., 1992)**.** Antibodies used can be either polyclonal or monoclonal, with the former being a mixture of antibodies attacking multiple epitopes of the same biomolecule, while the monoclonal ones attack a single epitope. Essential differences between the polyclonal over the monoclonal antibodies are that polyclonal can be more sensitive and less specific than monoclonal (e.g. can cover broader taxa, due to attacking more antigens that may include conserved antigens throughout more strains), whereas monoclonal tend to be very specific and less sensitive (e.g. their sensitivity depends on the conservation of the single targeted antigen throughout the various strains). Over the years, this system has become very popular due to its great selectivity and long duration of interaction of target proteins with biotin. It is a simple, affordable and fast method that makes it possible to separate living cells from samples (Díaz-Flores et al., 2015; Wang et al., 1992).

This method could lead to the establishment protocols of detection and isolation of *L. Pneumophilla* for everyday lab practice (Da Cunha et al., 2017).

A very positive development to that direction is the method of **Immuno-magnetic separation (IMS)**. This is a quick and easy technique that applies magnetic beads and a magnet to seperate a variety of targets, mostly cells, from a solution. There are hydrophobic surfaces that are covering the magnetic beads and they facilitate the binding of targets like antibodies and streptavidn/avidin (Wang et al., 1992). At first it was used for eukaryotic cells while in the last decades , it is used for for bacteria and viruses with promising results (Díaz-Flores et al., 2015; Wang et al., 1992).

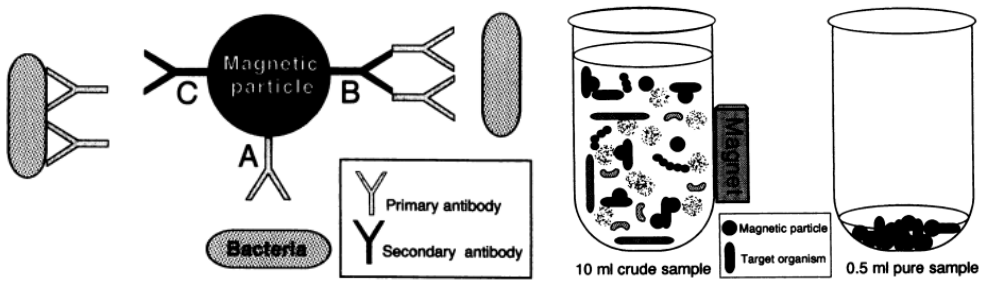


Figure 5. Immuno-magnetic separation (IMS) method procedure.

1.2. Aims of the thesis

The aims of the experiments presented in this thesis were: (i) the development a qPCR based culture independent protocol for the monitoring of the populations of the *Legionella* spp. and *L. pneumophila* in aquatic samples; (ii) and the assessment of the temperature variation effect on their population abundance and diversity in Pineios river.

2. Materials and methods

2.1 Experimental design

In the wider framework of the experimental procedure, two experiments were conducted. The first had the aim of the development of qPCR methods and the testing of them in environmental samples taken from Pineios River in Larissa. The sampling locations had the following coordinates (39.642524 N, 22.412692 E) for the Pineios River. The second experiment had the aim of implementation of these methods in environmental samples taken from Karla Lake with the subsequent development of an immunoprecipitation protocol for the detection of *Legionella* spp. and *L. pneumophila* in these samples. The reason behind the option of this microorganism for our experiments is the fact that it is a putative environmental pathogen and not of strictly clinical significance and its epidemiological aspects in the Pineios river have not been studied extensively. The days of the sampling for the samples of Pineios River were: 05/06/2023, 26/06/2023, 27/06/2023, 28/06/2023, and 04/07/2023 and the sampling volumes were 500 ml, 300 ml, 250 ml, 300 ml, and 800 ml respectively (~ 2,3 L collectively). From these samplings, 13 samples of concentrated bacteria occurred, after filtration (around 175 ml of water filtered for the concentration of bacteria in 1 sample). The filtration occurred either with the use of 0.22 μm pore filters or mixed cellulose esters (MCE) filters and dilution in 1X PBS, while DNA extractions were also performed. For the development of the qPCR methods for our markers we transformed *E.Coli* bacteria. These were cultured in LB-agar and the isolation of the recombinant plasmids was performed. After that we Sanger sequenced those plasmids for validating that their inserts belonged to the intended primer targets.

The bodies of water for the sampling were opted for their significance for the wider region of Thessaly. We opted that our sampling dates were in the summer as it is known that temperature is a really important positive factor in the growth of *L. pneumophila* as discussed in the introduction. Through the different places and dates of sampling, an assessment of spatial and temporal variance of environmental populations of *Legionella pneumophila* was possible, a result that is important for the monitoring of the population of this environmental opportunistic pathogen. The current thesis is relative to the first experiment.

2.2 Cell collection and enrichment

The cell collection methods to be considered tested were the: (i) water filtering followed by back-flashing, and (ii) centrifugation. In our experiments before the filtration of the cells the samples were sonicated 3 times for 15 sec so the solid particles will precipitate to the bottom. In our experiments we attempted two different approaches for extracting the cells for downstream applications. (i) The first one was filtering through a triple Whatman qualitative filter paper (diameter 110 mm and retention of 8-12 µm) (Friesenette, Knebel, Denmark), was employed for solid particle removal from the sample. Following a 0.22 µm-pore polyetherosulfone (PES) syringe-filter (Biomed Scientific, California, USA) was used for bacterial cell (including *L. pneumophilla)* enrichment. The filtering was achieved with the loading in a sterile syringe 50 ml (Shanghai Channelmed Import & Export Co. Ltd, Shanghai, P.R.China) and then passing of the water from the filter. This was repeated as many times necessary for all the sample to pass through the filter. We tapped the cells (≈ 2 taps/sec for 30 sec) and backflashed them in 1.5 ml of phosphate 1x buffered saline (PBS) pH 7.0 isotonic solution in 2 ml microcentrifudge tubes. The cells were stored at 4˚C for downstream analysis.

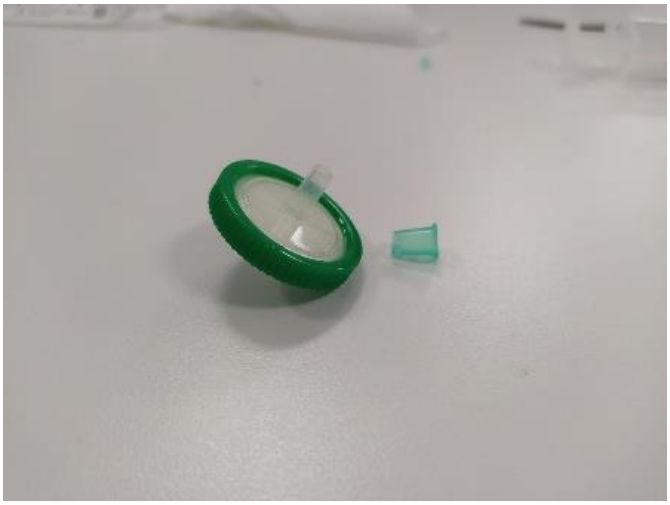


Figure 6. PE 0.22 µm pore syringe filter.

(ii) The main method of filtering was the employment of membranes from mixed cellulose esters (MCE) (Hawach Scientific Xi'an City, Shaanxi Province, P.R.China) for the enrichment of our samples. The membranes were inserted in an appropriate adapter device and 20-50 ml of sample were passed from the filter. This was repeated as many times as necessary for all the sample to flow through the membrane. After that the membranes were carefully removed from the adapter device with sterilized tweezers, folded and put on either a 2 ml microcentrifudge tube along with 1.5 mL of PBS or in a Power Bead Pro Tube of DNeasy®PowerSoil® Pro Kit (Qiagen, Düsseldorf, Germany). Except from the use of syringes in some cases a pump was used for the cell enrichment.

Figure 7. MCE membrane of 0.22 μm pores and 200 mm diameter.

2.3 Media and strains

In our method setup two bacterial strains have been used:

- *E.coli* DH5a.

- *L. pneumophila* serogroup 1 NCTC 11192.

The *E.coli* strain was used as a carrier for the recombinant plasmids used as standards for the qPCR methods. They carry a mutation that allows the differentiation between blue and white colonies. Also their genome contains genes for the recombinase RecA1 of the RecA group of recombinases ensuring the stability of the insert. Additionally, the strain possess a protein that inhibits the enzymatic activity of endonuclease EndA, increasing the in that way the efficiency of the transcription of the vector (Selvarasu et al., 2009).

The *E.coli* strain was used as a carrier for the pGEM T easy plasmid vectors used in the prep of the standards for the employed qPCR methods. The aforementioned plasmid vectors carry the *lacZ* gene coding for β-galactosidase. Once inserts integrate within the gene generating recombinant plasmids, the *lacZ* function is disrupted. Transformed recombinant vectors into E. coli give normal white colonies, whereas, non-transformed vectors not receiving an insert (aka have functional *lacZ* genes), give blue colonies due to the tracing of the chromogenic (blue-pigment producing) β-galactosidase mediated hydrolysis of 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-gal) after the induction of the expression of *lacZ* with isopropyl beta-D-1-thiogalactopyranoside (IPTG)(Kostylev et al., 2015)

The *L. pneumophila* strain was a donation of the ‘’Hygiene and Epidemiology’’ laboratory of the School of Medicine of University of Thessaly, Professor Christos Hadjichristodoulou and attending Doctor Maria Kyritsi. The strain was acquired from LGC Ltd. (Teddington, England, UK).

The media that these bacteria were cultured were Louria-Bertani Broth (in liquid cultures), Louria-Bertani Broth agar (in solid cultures) for *E.coli* and Buffered Charcoal Yeast Extract (BCYE) agar for *L. pneumophila.*

The ‘’Louria-Bertani’’ media were prepared in the laboratory while the Buffered Charcoal Yeast Extract (BCYE) agar were purchased (Thermo Scientific Cat# PO5072A). There have been used 500 ml of Louria Bertani Broth (LB- Broth) and 500 ml Louria-Bertani Broth (LB- Broth) with adding of D-glucose. These were used also 500 ml of LB-agar for the creation of solid media in petri dishes. The consistency of each of the LB-media can be seen in the following tables.

Table 4. Consistency of LB-Agar.

Luria-Bertani agar	
Agar	7.5 gr
Bacto-tryptone	5 gr
Bacto-yeast extract	5 gr
NaCl	2.5 gr

Table 5. Consistency of LB-Broth.

Luria-Bertani broth	
Bacto-tryptone	5 gr
Bacto-yeast extract	5 gr
NaCl	2.5 gr

Table 6. Consistency of ''SOC'' medium.

Luria-Bertani ‘’SOC’’	
D-Glucose	1.8 gr
Bacto-tryptone	5 gr
Bacto-yeast extract	5 gr
NaCl	2.5 gr

The incubation of the *E.coli* bacteria was for 24h at 37 °C or at room temperature for 3 days.

The consistency of Buffered Charcoal Yeast Extract (BCYE) of one can be seen in the following table.

Table 7. Constitution of Charcoal Yeast Extract (BCYE).

Ingredients	**g/L**
Yeast extract	10.0
ACES Buffer	10.0
Active Carbon	2.0
KOH	2.8
a- ketoglutarate	1.0
L-cysteine	0.4
Iron pyrophosphate $Fe_4(P_3O_7)_3$	0.25
Agar	12.0

2.4 DNA extraction

The extraction was performed according to DNeasy®PowerSoil® Pro Kit (Qiagen, Düsseldorf, Germany) protocol. The basic steps for the DNA extraction in all protocols are the following:

- Lysis of the membranes of the cells and release of their content. This is achieved with either the use of specific solutions that contain surfactants or through mechanical lysis with the use of beads.
- Denaturation of the proteins of the cells with the use of proteolytic enzymes. Through the above method, the inactivation of nucleases that could compromise DNA integrity is achieved. Also in this step, the DNA integrity is achieved. Also in this step, the conditions are set for facilitating the binding of the DNA in the silica membrane in the downstream steps.
- Separation of the DNA from the proteins. This is achieved with the use of salt solutions of high ionic power and absolute ethanol or with the binding in magnetic beads
- Clean up of the DNA from residuals of salts and ethanol.
- Regain of pure DNA in a weak alkaline solution (pH 8-9) that contains EDTA. EDTA is a chelation factor that binds in ions, protecting the DNA from precipitation and potential nuclease activity (Papanikolaou et al., 2016b).

2.5 Quantitative PCR (qPCR) method setup

In order to setup the qPCR reactions, we have amplified the target inserts at DNA extracts of environmental samples, verified the insert sequencing the inserts of the resulting primers, and set the associated standard curves up and tested the qPCR reaction efficiencies.

PCR amplification setup of environmental DNA targets

Temperature gradient polymerase chain reactions (PCR) were conducted for optimising the reactions for primer sets *LEG*, *L5S* and mip. We also tested the primer sets that we used for the total bacterial copies of the 16S rRNA gene. The tested primers are provided in Table 13 at the results section along with the optimised PCR conditions. The information about the reaction mixtures is provided in table 8 below.

Table 8. Volumes of each of the reagents in different final volumes of reactions.

Reagent	**Concentration (final)**
10 X buffer A (contains $MgCl_2$ at 25 mM)	2.5 Mm
dNTPs (200 mM each)	4 mM
Taq (5 U/µl)	0.02 U/µL
F prim (10 µM)	0.4 µM
R prim (10 µM)	0.4 µM
BSA (10 µg/µl)	0.5 µg/µl

Table 9. Final quantities of reagents in PCR reactions.

Reagent	**Volume (in µl)**	**Volume (in µl)**	**Volume (in µl)**
H_2O	12.92	33.8	64.6
10 X buffer A (contains $MgCl_2$ at 25 mM)	2	5	10
dNTPs (200 mM each)	0.4	1	2
Taq (5 U/µl)	0.08	0.2	0.4
F prim (10 µM)	0.8	2	4
R prim (10 µM)	0.8	2	4
Template	2	5	10
BSA (10 µg/µl)	1	1	5
Total (µl)	20	50	100

PCR product cloning and transformation

The PCR products were analysed in 1.5% w/v agarose gels. Following that, the products of that polymerase chain reaction were gel-excised and cloned in suitable vectors. The vector used was pGEM® - T Easy (Promega Corporations). 1 µl 10 X T4 DNA ligase bugger (ThermoFisher Scientific) along with T4 ligase (5U/µl, ThermoFisher Scientific), 1 µl DNA-insert and ddH_2O were added to fixed volume of 10 µl. The optimal ratio of molecules insert/plasmid vector was ¼ to 4 according to manufacturers' instructions. The ''ligation'' reactions were incubated overnight at 4 °C.

The ligation products were transformed in competent *E.Coli* DH5a cells with heatshock. After that, in solid (Luria-Bertani agar) and liquid (Luria-Bertani broth) media. The plasmids were extracted with

the protocol of a kit Nucleospin®Plasmid (Macherey-Nagel, Düren, Germany). After the extraction, the plasmid insert were Sanger sequenced to confirm the existence of the sequence of interest.

The insert containing plasmids were used for generating qPCR standards as follows. The extracted plasmid DNA with the largest concentration was selected for this purpose for each set of primers. The extracted plasmid DNA with the largest concentration was selected for this purpose for each set of primers. After that,the calculation of copies for each DNA of interest occurred using the following formula: Number of copies= (ng X [6 (ng × [6.022×1023])/(length ×[1×109]×650). From which:

- ng is the amount of DNA (plasmid, primer, etc.) you have in nanograms.
- 6.022 x 10^{23} = Avogadro's number.
- Length is the length of your DNA fragment in base pairs. Just multiply by 1000 if you are working in kb.
- We multiply by 1 x 10^{9} to convert the outcome to nanograms.

qPCR reactions optimization and performance

qPCR reaction optimization was performed regarding the number of cycles (reflecting the target rarity) and the primer hybridization temperatures. The qPCR reactions were performed with the KAPA SYBR® FAST mix (KAPA SYBR® FAST,Roche Basel, Switzerland) where the following cycling conditions were employed: 95 °C for 3 mins for initial enzyme activation; And at each cycle 95 °C for 30 sec of denaturation were followed by 55 or 60 °C (depending on the primer set) for 30 sec for primer hybridization and 72 °C for 40 sec for elongation; 10 min at 72 °C for final elongation. The reactions consisted of 35-40 cycles total.

After that, serial ten0fold dilutions were prepared of the plasmid extracts (9 µl of of ddH_2O and 1 µl of solutions of the plasmids) at plasmid concentrations as low as 100 copies. The highest plasmid concentrations were 10^{10} per µl. These were used to build standard curves for our qPCR reactions. Following that, the master mix for the qPCR reactions was prepared. The constitution of the master mic for one reaction can be seen on the following table.

After that, serial ten-fold dilutions were prepared of the plasmid extracts (9 µl of ddH2O and 1 µl of solution of the plasmids), at plasmid concentratios as low as 100 copies per µl. The highest plasmid concentrations were 10^{10} per µl. These were used to build standard curves for our qPCR reactions. Following that, the master mix for the qPCR reactions was prepared. The constitution of the master mix for one reaction can be seen in the following table.

Table 10. Reagents and their respective volumes in a 10 µl qPCR reaction.

Reagents	Volume in µl
Sybr-Green	5
Forward primer (10 µM)	0.1
Reverse primer (10 µM)	0.1
H_2O	3.6
BSA (20 mg/ml)	0.2

In all PCR reactions (BSA Bovine Serum Albumine) have been used to increase the efficiency of the reactions, as this molecule deters the interactions of chelate compounds with the polymerase (Kreader, 1996). The final volume qPCR reaction used was 10 µl. The qPCR were run including the samples and the standard curves (triplicates) along with the negative controls.

Except for our three primer sets *LEG, L5S* and *mip*, a qPCR reaction for the *16S* gene have been conducted with the use of 2 primer sets (the 16 S rRNA gene is a marker of phylogenesis/biodiversity of bacteria) for expressing our results per 16S rRNA gene copy. This way we could express our *Legionella* marker gene counts in the context of the total bacterial community 16S rRNA gene counts.

2.6 Microbiome diversity analysis

DNA extracts were also shipped for sequencing of the 515f/806r primer set targeting the total prokaryotic 16S rRNA gene generated amplicons. The retrieved sequences were analyzed with dada2 v1.22 (Callahan et al., 2016) and the phyloseq v1.38 (McMurdie & Holmes, 2013) packages in R 4.1.3 (R Core Team, 2023).

3. Results

3.1 Cell concentration method results

The tested cell concentration protocols included 0.22 μm-pore polyetherosulfone (PES) syringe-filters. The cells filtered were dislodged with sonication along with back-flashing. We knew from literature that this was an efficient method of concentration of bacterial cells. The use of mixed cellulose esters (MCE) filters were also used along with sonication. Out of the samples of the whole extracted DNA the first two came after the filtering with the use of polyetherosulfone (PES) syringe-filters and the remaining three with the use of mixed cellulose esters (MCE) filters. The DNA concentrations of each of the samples after the implementation of the DNA extraction protocol can be seen on the next table (Table 11.)

Table 11. The DNA concentrations of each of the samples after the implementation of the DNA extraction protocol The mean quantity of DNA retrieved with each method can be seen in the last column of the table.

treatment	Rep	Abs260	Abs280	260/280	Concentration (ng/ul)	Mean (ng/μl)
PES syringe	1	0.098	0.08	1.23	4.9	3.6±1.3
PES syringe	2	0.073	0.042	1.73	3.6	
PES syringe	3	0.046	0.033	1.37	2.3	
MCE manifold	1	0.145	0.097	1.5	7.3	7.55±0.35
MCE manifold	2	0.156	0.105	1.48	7.8	

Each of the '' PES syringe'' sample corresponds to the Peneios_Sample_i_and Peneios_Sample_ii_, respectively. The '' MCE manifold'' samples correspond to Peneios_Sample_iii_ and Peneios_Sample_iv_ and Peneios_Sample_v_ respectively. The results were acquired with the use of 2 μl of each extracted sample after spectrophotometry in the Thermo Scientific™ NanoDrop™ 2000/2000c (ThermoFisher Scientific).

3.2 Primer testing and PCR product evaluation

PCR results led to the conclusion of the successful amplification of the expected products: namely 654 bp for the LEG primer set, 104 bp for the LS primer set and 115 bp for the *mip* targeting primer set in different temperatures (Figures 8 and 9). The optimal temperatures for the PCR reactions were 56 °C for the first primer set and 60 °C for the other two (Table 10).

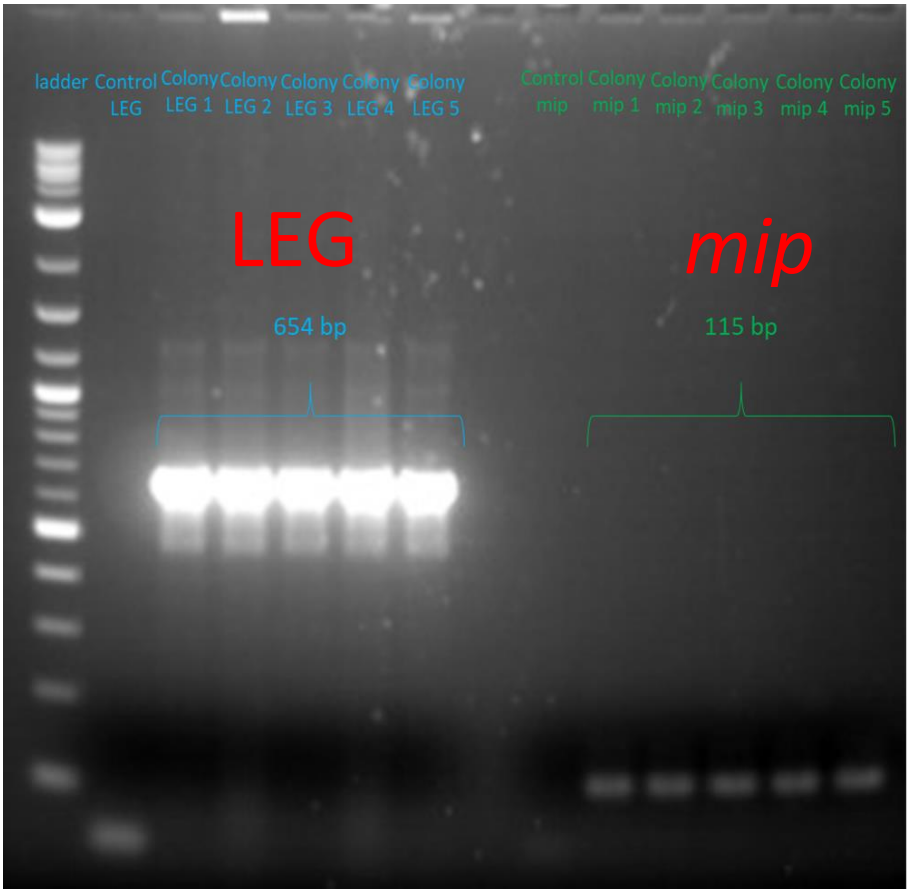
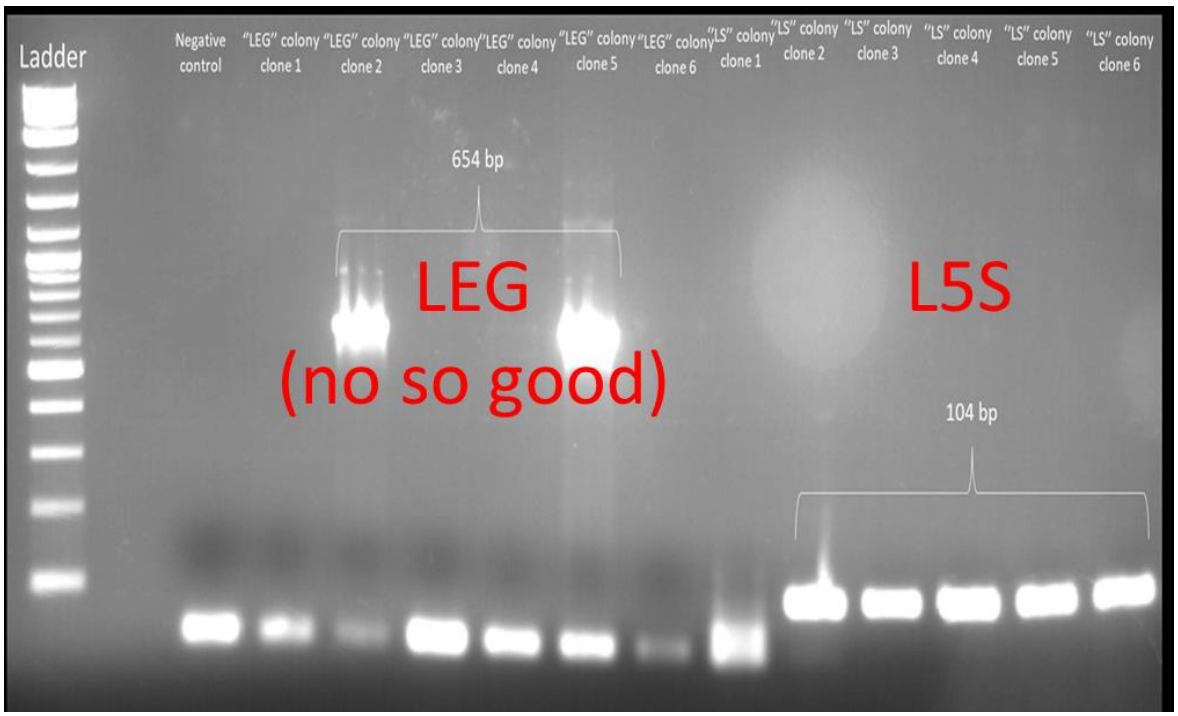


Figure 8. Products of primer sets LEG and mip of colony PCR after the transformation of E.Coli with their products.

Figure 9.Products of primer sets LEG and l5S labelled '' LS'' of colony PCR after the transformation of E.Coli with their products.



Based on these results, 50 µl PCR mixtures were prepared for each primer set with *L. pneumophila* derived templates. The PCR products were used for cloning and insert sequencing for assessing whether the PCR targets were the intended ones. Sequencing results validated the product specificity according to the results of their comparison with the National Center for Biotechnology Information (NCBI) databases (*LEG* (amplifying genes that code for the *16S* rRNA protein of the *Legionella group*), *L5S* (amplifying genes that for code the *5S* rRNA protein of the *Legionella pneumophila*) and MIP (amplifying genes that code for the macrophage infectivity potentiator with *L. pneumophila).*

Table 12. The various primer set for Legionella pneumophilla, along with the consensuses of the sequences products in the environment and the best results after BLAST.

Primers	Product Sequence	Best ''Hit'' in Reciprocal Blast
1st Legionella set: Targeting the *16S* rRNA gene of *Legionella* spp.		
LEG	AAGATTAGCCTGCGTCCGATTAGCTAGTTGGTGGGGTAAGGGCCTACC AAGGCGACGATCGGTAGCTGGTCTGAGAGGATGACCAGCCACACTGG AACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT ATTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAA GAAGGCCTGAGGGTTGTAAAGCACTTTCAGTGGGGAGGAGGGTTGA TAGGTTAAGAGCTGATTAACTGGACGTTACCCACAGAAGAAGCACCG GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTT AATCGGAATTACTGGGCGTAAAGGGTGCGTAGGTGGTTGATTAAGTTA TCTGTGAAATTCCTGGGCTTAACCTGGGACGGTCAGATAATACTGGTT GACTCGAGTATGGGAGAGGGTAGTGGAATTTCCGGTGTAGCGGTGAA ATGCGTAGAGATCGGAAGGAACACCAGTGGCGAAGGCGGCTACCTG GCCTAATACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGA TTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCAACTAGCTGTTGG TTATATGAAAATAATTAGTGGCGCAGCAAACGCGATAAGTTG	*Legionella pneumophila* strain ERS 1305867 chromosome, complete genome
2nd Legionella set: Targeting the *5S* rRNA gene of *Legionella* spp.		
L5	GCGATGACCTACTTTCGCATGAGGAAGCCTCACACTATCATTGGCGCG GAAATGTTTCACTTCTGAGTTCGAGATGGTATCAGGTGGTTCCAAATC GCTATAGT	*Legionella pneumophila* strain ERS 1305867 chromosome, complete genome
3rd Legionella set: Targeting the *mip* gene of *L. pneumophila*		
MIP	CCAATTGAGCGCCACTCATAGCGTCTTGCATGCCTTTAGCCATTGCTTC CGGATTAACATCTATGCCTTGATTTTTAAAATTCTTCCCCAAATCGGCAC CAATGCTATAAGACAA	*Legionella pneumophila* strain ERS 1305867 chromosome, complete genome

Table 13. The various '' Legionella'' primer sets used in our experiments with the conditions of the gradient PCR along with their optimal annealing temperature and the sequences of the primers.

Primers	Sequence	PCR conditions
- 1st Legionella set: Targeting the *16S* rRNA gene of *Legionella* spp.		
LEG-225	AAGATTAGCCTGCGTCCGAT	Polymerase activation at 94°C for 5 min, followed by 40 cycles with denaturation at 94°C for 30 sec, hybridization temperature gradient at 52 °C – 56 °C for 1 min. Then the elongation of the new synthesized DNA chain at 72**°**C for 40 s and then final elongation at 72°C for 5 min. 4 °C infinitely. Optimal annealing temperature at 56 °C.
LEG-858 (Qin et al., 2012)	GTCAACTTATCGCGTTTGCT	
2nd Legionella set: Targeting the *5S* rRNA gene of *Legionella* spp		
L5SL9	ACTATAGCGATTTGGAACCA	Polymerase activation at 95°C for 5 min, followed by 40 cycles with denaturation at 95°C for 10 sec, hybrization temperature gradient at 55 °C-60 °C for 1 min. Then the elongation of the new synthesized DNA chain at 72**°**C for 40 s and then final elongation at 72°C for 5 min. 4 °C infinitely. Optimal annealing temperature at 60 °C.
L5SR93 (Qin et al., 2012)	GCGATGACCTACTTTCGCAT	
3rd Legionella set: Targeting the *mip* gene of *L. pneumophila*		
MipF	TTGTCTTATAGCATTGGTGCCG	Polymerase activation at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 15 sec, hybridization temperature gradient at 55 °C-60 °C for 1 min. Then the elongation of the new synthesized DNA chain at 72**°**C for 40s and then final elongation at 72°C for 5 min. 4 °C infinitely. Optimal hybridization temperature at 60 °C.
MipR (Collins et al., 2015)	CCAATTGAGCGCCACTCATAG	

Table 14. ''General Baceteria'' 16S primer sets used for expressing our Legioenlla marker gene counts in the context of total bacterial community.

Primers	Sequences	PCR Conditions
General/ Total bacteria primer sets (16S rRNA gene):		
338 f	CCTACGGGAGGCAGCAG	Polymerase activation at 95°C for 3 min, followed by 35 cycles with denaturation at at 95°C for 30 sec, hybridization of primers and their target at 55°C for 30s . Then the elongation of the new synthesized DNA chain at 72°C for 40s and then final elongation at 72°C for 10 min. 4 °C infinitely.
518 r (T. Liu et al., 2017)	ATTACCGCGGCTGCTGG	
8 f	CACGGATCCAGACTTTGATYMTGGCTCAG	Polymerase activation at 95°C for 3 min, followed by 35 cycles with denaturation at at 95°C for 30 sec, hybridization of primers and their target at 55°C for 30s . Then the elongation of the new synthesized DNA chain at 72°C for 40s and then final elongation at 72°C for 10 min. 4 °C infinitely.
1512 r (Barbosa et al., 2016)	ATTACCGCGGCTGCTGG	

3.3 Final qPCR method setup

After the execution of qPCR each of the primer sets, the amplification plot and the standard curves of each of them were checked for the efficiency and the repeatability of the results reactions. qPCR efficiencies were within acceptable ranges according to the generated standard curves with plasmids containing environmental DNA products of these primers as inserts. Standard curves had efficiencies between 80% and 110% with coefficient of determination values R^2 of > 0.95. Indicatively the standard curve plot of the qPCR for the *mip* and *L5S* targeting primer sets can be seen here (Figures 10,11,12).

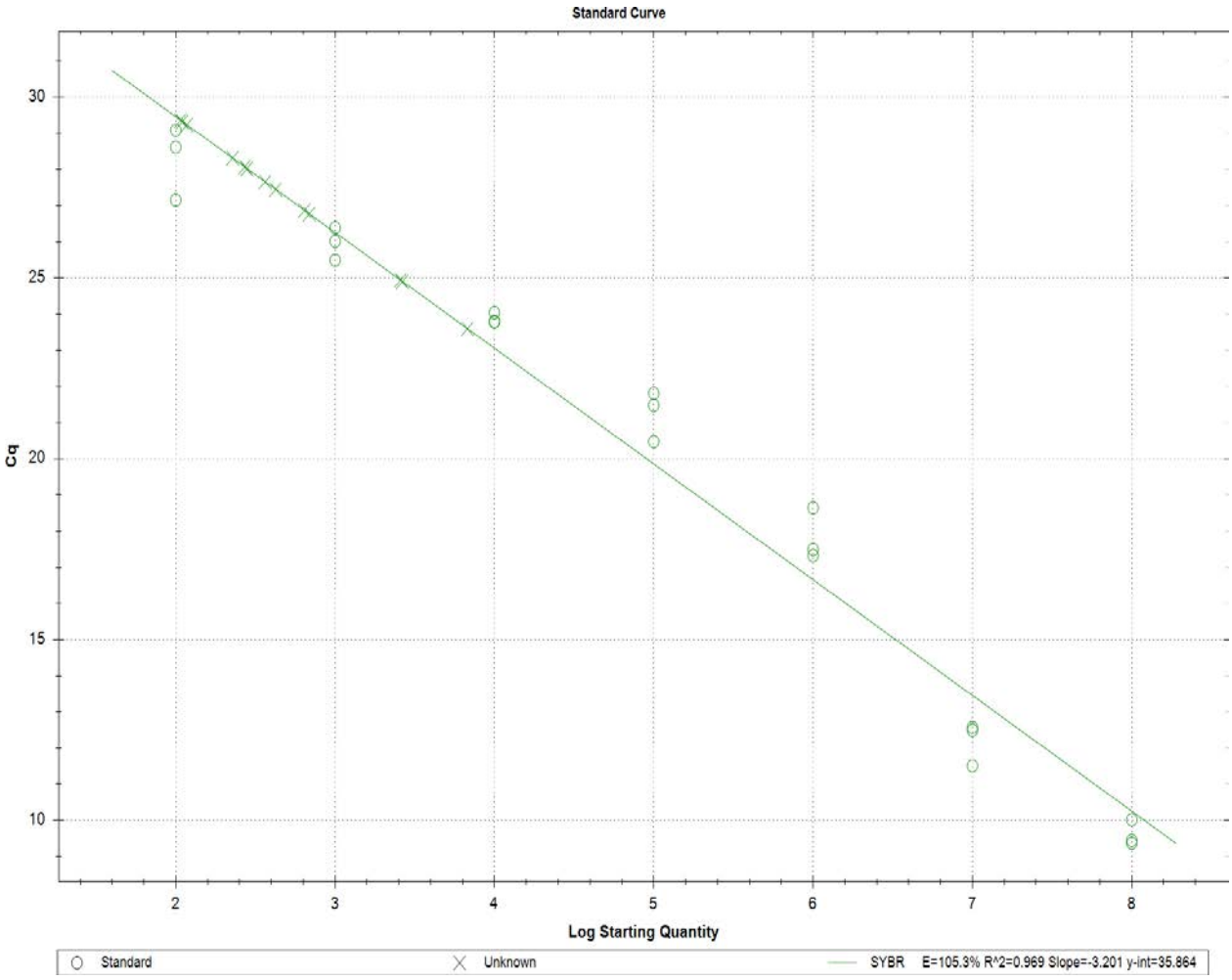


Figure 10. Standard curve plot of the the qPCR of the mip gene.

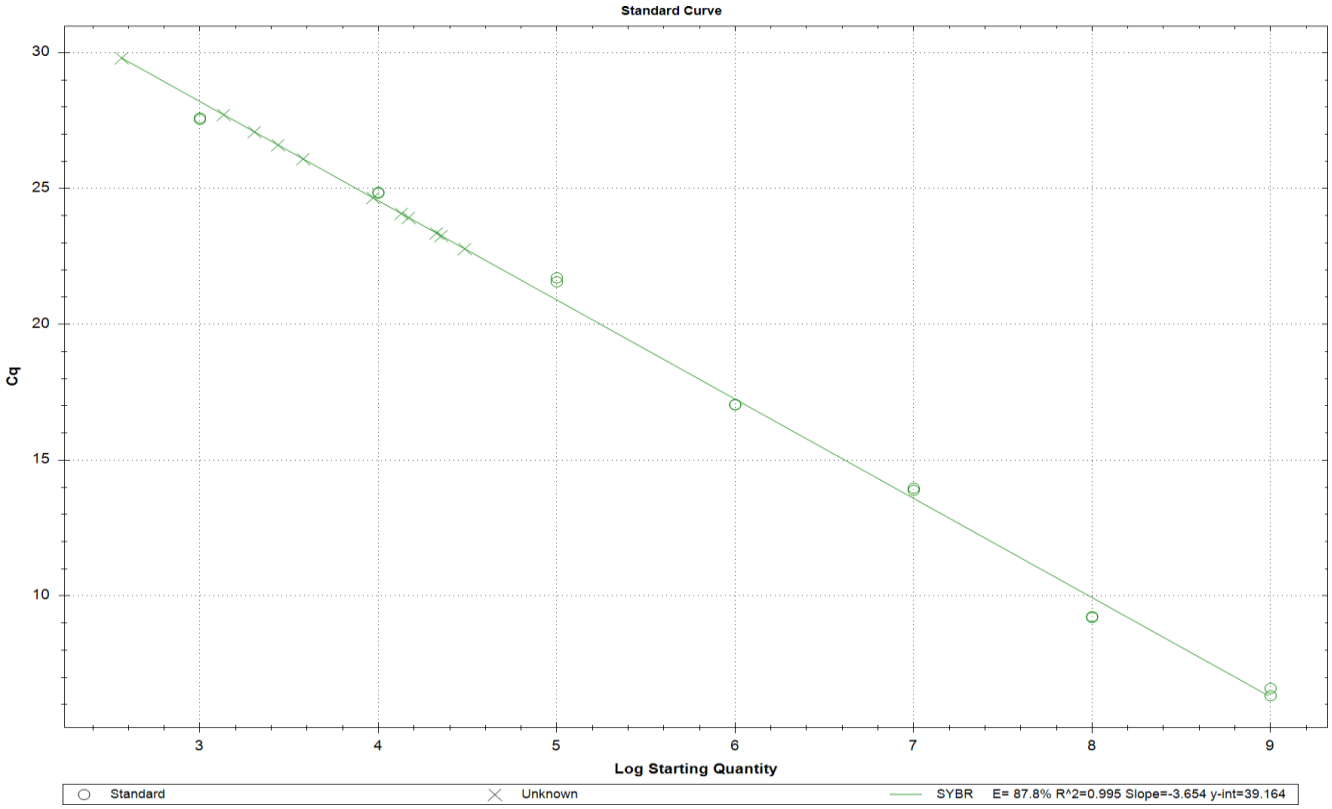


Figure 11 Standard curve plot of the qPCR of L5S primer set..

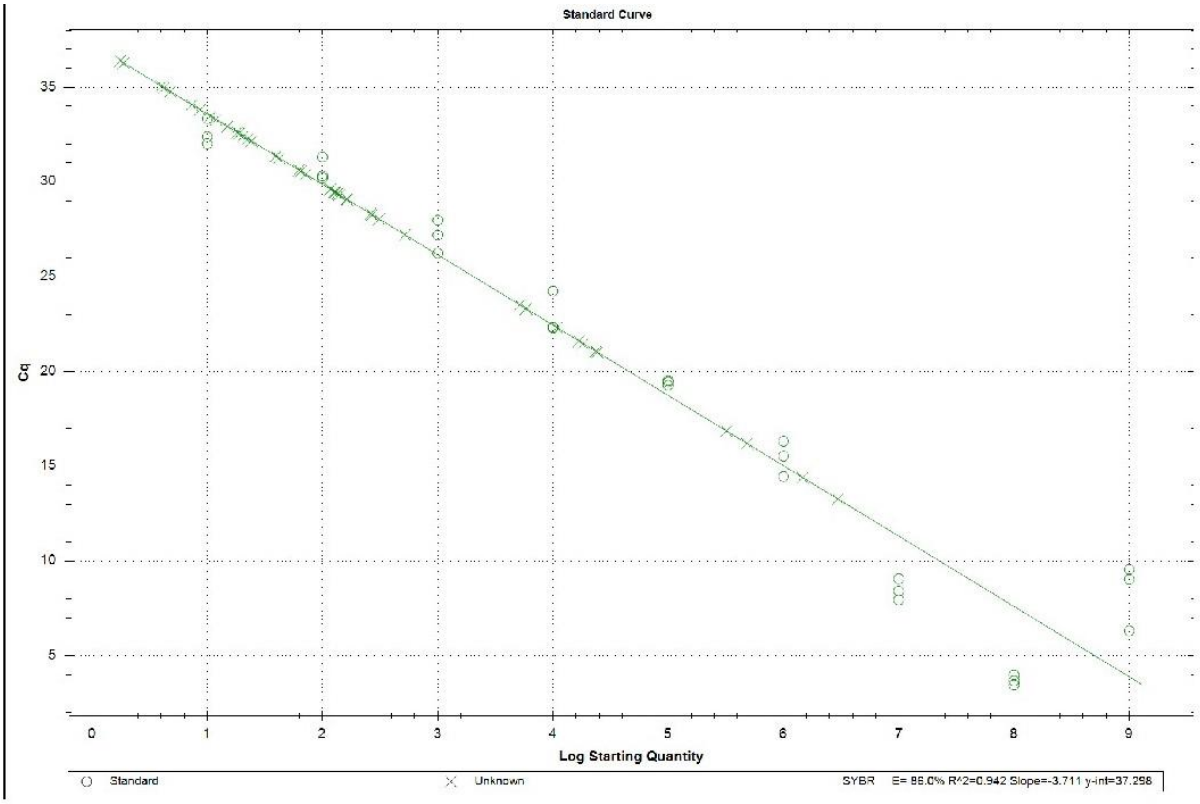


Figure 12 Standard curve plot of the qPCR of LEG primer set.

3.4 Temporal variation of populations

After setting up the methods we proceeded with the analysis of the environmental samples as follows. From the 13 samples that were collected after filtering from the days of the sampling, 3 were collected at 05/06/2023, 2 at 26-27-28/06/2023 and 4 at 04/07/2023. From those, 1 of the samples collected at 05/06/2023, 2 samples collected at 27-28/06/2023 and 2 samples collected at 04/07/2023 were firstly filtered, in order to retrieve concentrated the bacterial cells out of ml of sample, and then were used for extracting the cell DNA. These samples were named: Peneios_Sample_i_, Peneios_Sample_ii_, Peneios_Sample_iii_ and Peneios_Sample_iv_ and Peneios_Sample_v_, for the 05/06/2023, 27-28/06/2023 and 04/07/2023 respectively. They can be seen in the graphs. In the graphs converted into copies per ml quantification of the samples from River Pineios can be seen along with the abient temperatures as retrieved from close-by meteorogical stations. Highest and lowest temperatures (34 ˚C and 29 ˚C) (Figures 13., 14., 15., 16.). Overall, the results show increasing trend for total bacteria and *Legionella* spp. based on the total bacterial 16S rRNA gene and the ribosomal gene targeting *Legionella* spp. specific primers (with *Legionella* spp. 16S rRNA gene targeting primers showing a clear monotonic pattern; (Figures 13,14 and 16). The *L. pneumophila* specific *mip* targeting primers showed a temperature/time inconsistent pattern (Figure 16).

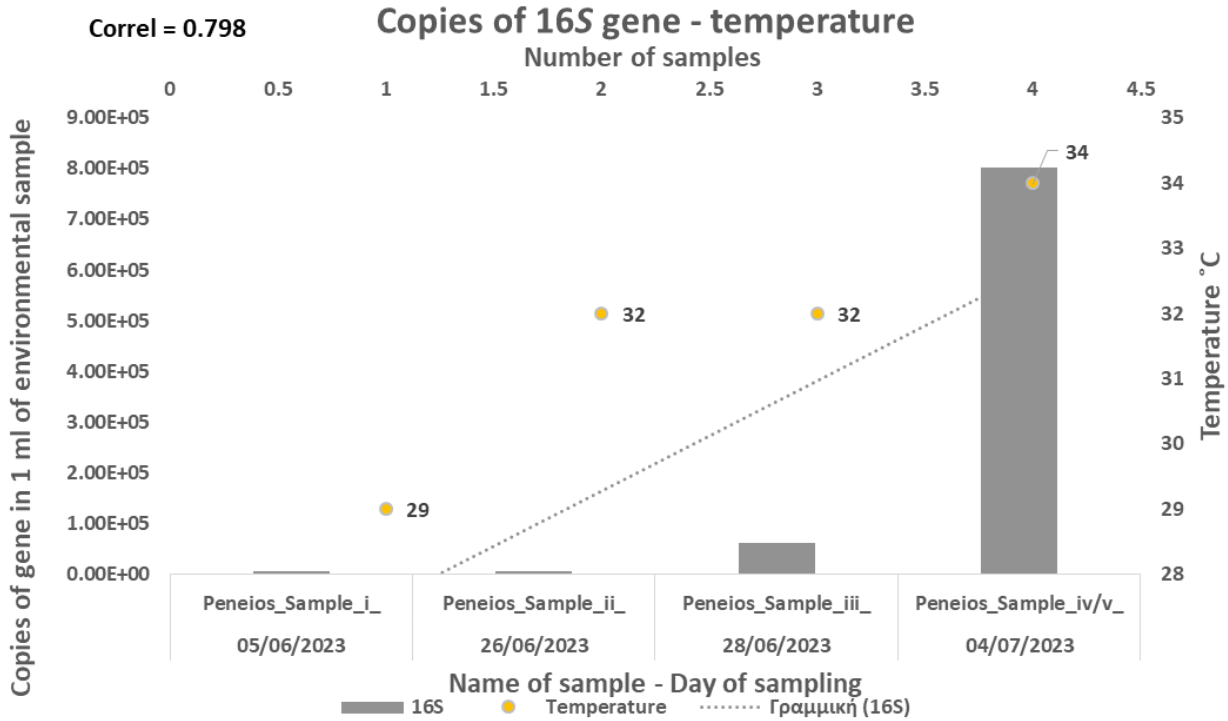


Figure 13. qPCR results of the 16S gene copies at Pineios samples with the corresponding temperatures of the sampling times. Linear regression analysis is also provided for roughly assessing the trends of the counts along time. Also correlation coefficient is show for assessment of linear correlation between the copies and the temperature variance.

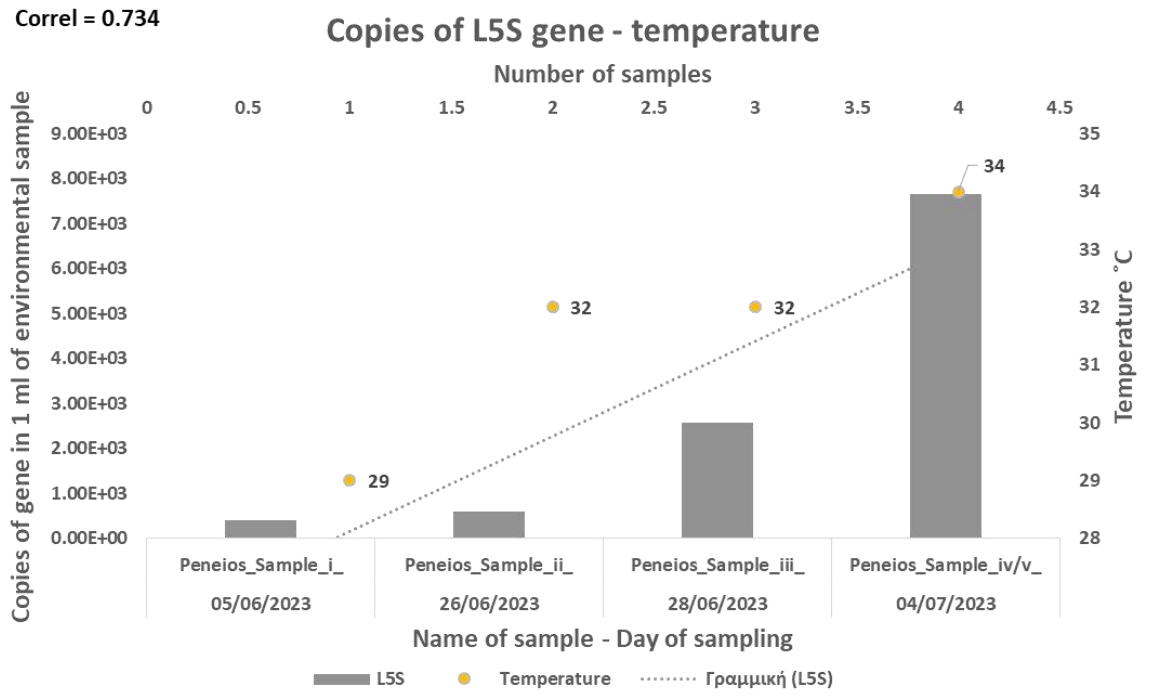


Figure 14. qPCR results of the L5S gene copies at Pineios samples with the corresponding temperatures of the sampling times. Linear regression analysis is also provided for roughly assessing the trends of the counts along time. Also correlation.

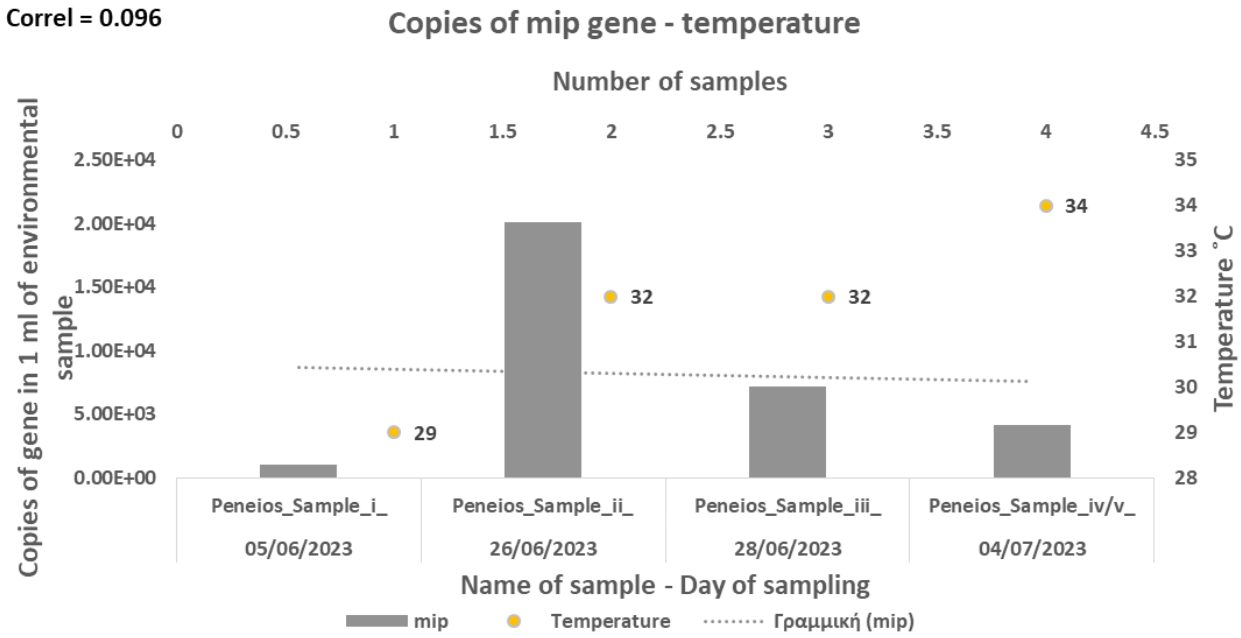


Figure 15. qPCR results of the mip gene copies at Pineios samples with the corresponding temperatures of the sampling times. Linear regression analysis is also provided for roughly assessing the trends of the counts along time. Also correlation coefficient is provided for assessment of the linear correlation between the copies and the temperature variance each of the days.

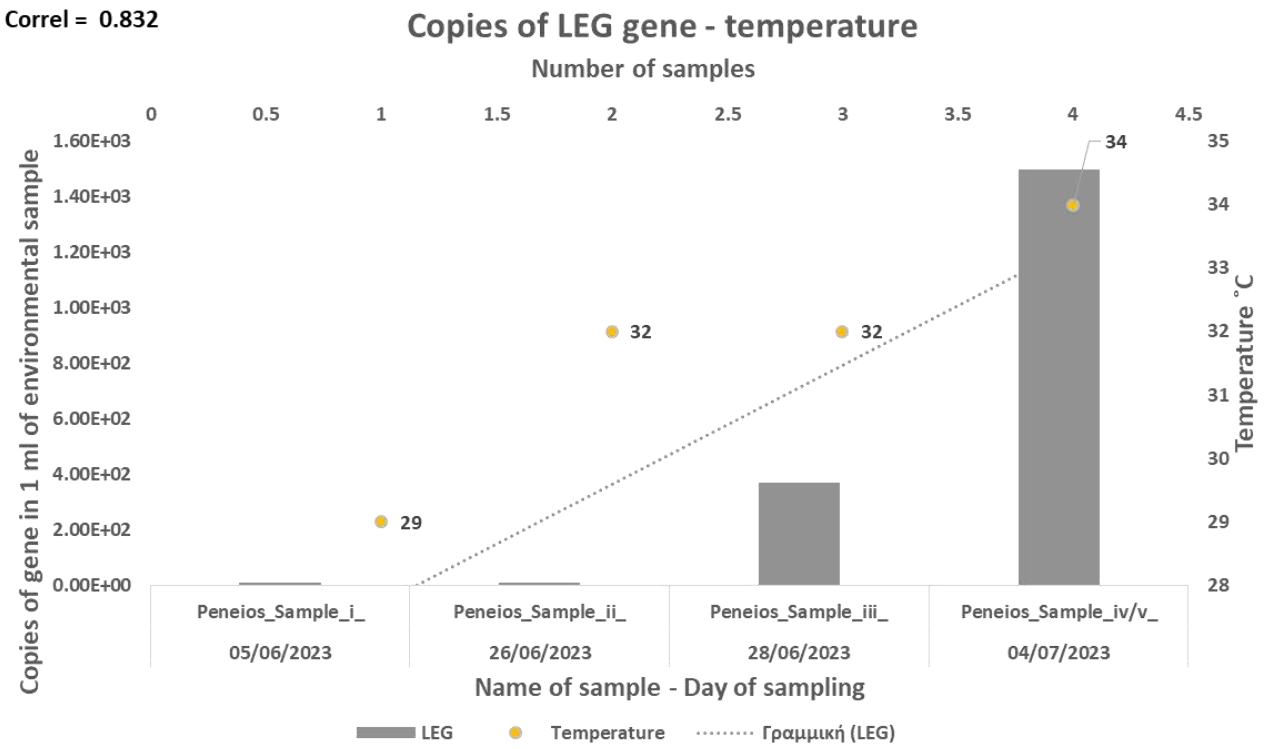


Figure 16. qPCR results of the LEG gene copies at Pineios samples with the corresponding temperatures of the sampling timesl. Linear regression analysis is also provided for roughly assessing the trends of the counts along time. Also correlation coefficient is provided for assessment of the linear correlation between the copies and the temperature variance each of the days.

3.5 Diversity analysis of the Pineios Samples

We observed a pattern of increase of diversity through time. According to the literature there are almost no studies of microbial diversity analysis of the Pineios River. Thus in our experiments we attempted to address that and acquire an idea of the possible diversity of the river. α-diversity showed an increase of the sample of Pineios with time in all the diversity indexes (Figure. 17)

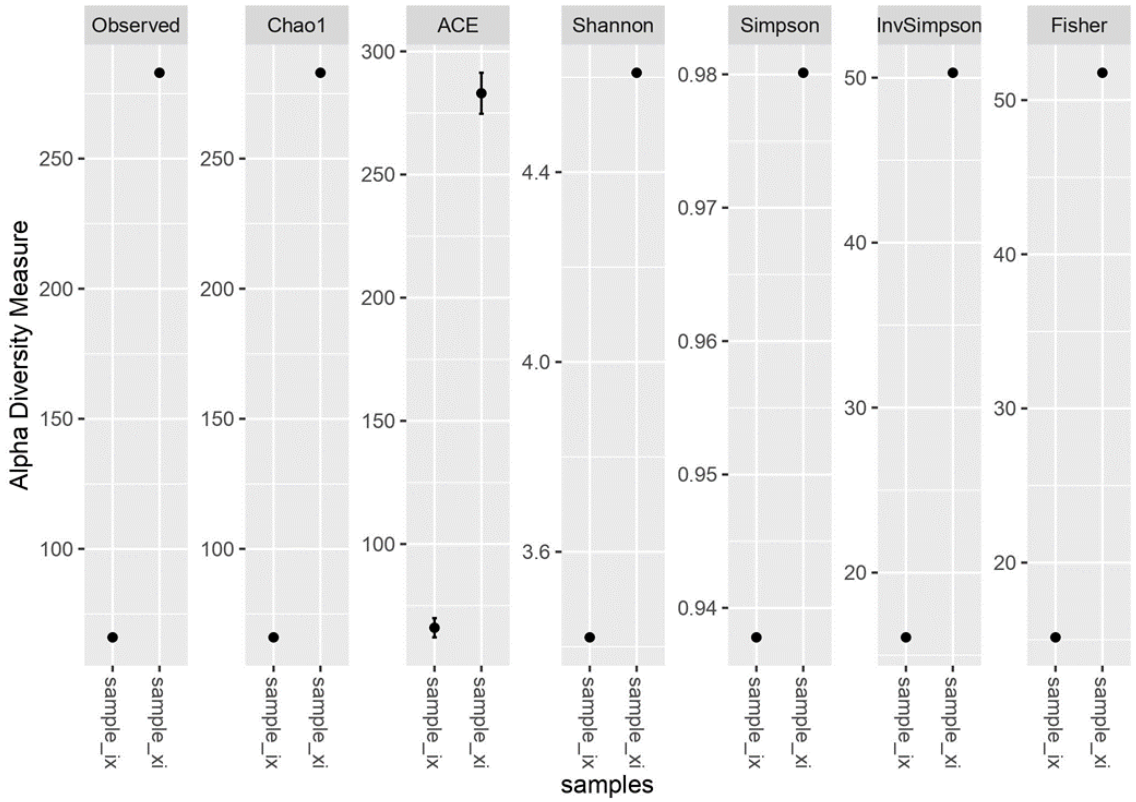


Figure 17. a-diversity indexes:a) Observed,b) Chao1 index- richness based, c)Abundance-based estimate predictors (ACE), d) Shannon index, e) Simpson index High abundance index, f)Inverse-Simpson index (InvSimpson) Index of intermediate abundance of samples, g)Fisher index-high abundance index. Samples: Pineios _ix and Pineios_xi. Dates of sampling: 28/06/2023 and 04/07/2023.

The differences in the composition of the samples have showed to follow a temporal pattern (Figure 18.). For example the phylum of *Actinobacteria* was reduced and was replaced partially by the class of *α-Proteobacteria* and genus of *Bacilli*. In addition, the class of *γ-proteobacteria*, where *L.pneumophila* belongs, was reduced in relative abundances, and especially regarding the taxa of *Pseudomonas* and *Pseudomonadaceae*. The genus of *Acinetobacter* increased in relative abundance with time. A great relative increase has been also documented in the genus of *Chloroflexia* and also other types of bacteria manifested tendencies of increase in the later sampling day.

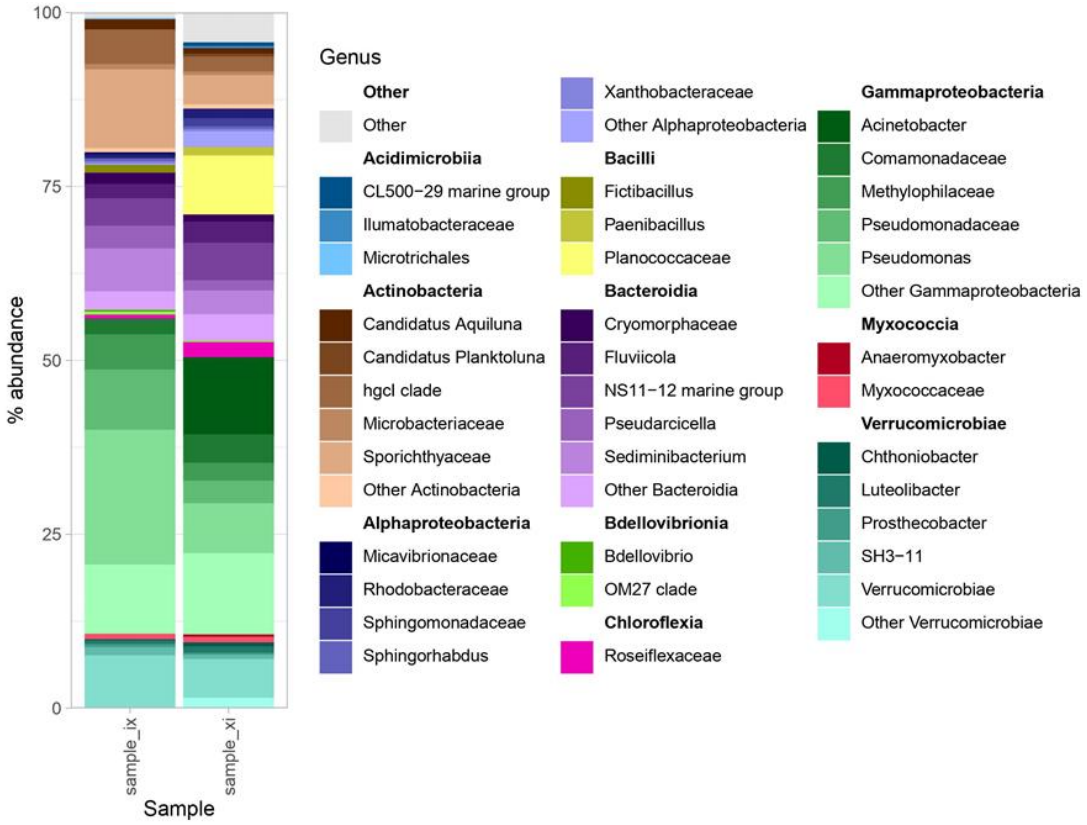


Figure 18. Stacked barplot of relative abundances of micrrorganisms of the samples. Samples: Pineios_ix, Pineios_xi, Dates of sampling: 28/06/2023, 04/07/2023

4. Discussion

4.1 Method setup

Our results suggested a successful setup of the qPCR-based methods for the quantification of the *Legionella* spp. and *L. pneumophila* in aquatic environments. Out of the tested cell concentration protocols one concerning filtered cells that were dislodged with sonication along with the use of mixed cellulose esters (MCE), seemed to be the most efficient. Filtration is known to be effective out of cell collection methods like e.g. centrifugation (Ezenarro et al., 2022; Y.-C. Kim & Morrison, 2009; Nnadozie et al., 2018), while sonication is considered to improve the efficiency of post filtering bacterial cell recovery (Nnadozie et al., 2018).

Out of the tested markers, the *mip* gene is *L. pneuomophila* specific while the other markers (LEG and L5S primer set targets) are more generic targeting other *Legionella* spp. or total bacteria (bacterial 16S rRNA and *Legionella* 5S rRNA gene specific). Depending on their specificity levels, these primers can be used in complementary fashion in environmental epidemiology studies (aka for different levels of alert). Plenty of studies focus on the use of single marker, while fewer of them combine markers together. For example, for monitoring *L. pneuomophila* in built or natural environments, markers like either the 16S rRNA gene (Ahmed et al., 2019) or the macrophage infectivity potentiator *mip* gene were used (Boss et al., 2018). Such studies contribute in

understanding the dominance of known pathogenic strains like *L. pneuomophila* over the rest, less virulent *Legionella* spp., in environmental gradients (Lu et al., 2015). For example, *L. pneumophila* responses to various concentrations of ions or disinfectants and the presence of other heterotrophic bacteria have been recorded in the past (Donohue, 2021), with most studies focusing in residential areas (Al-Matawah et al., 2012; Donohue, 2021).

4.2 Total bacterial, *Legionella spp.* and *Legionella Pneumophila* counts, and total prokaryotic diversity in Pineios river

Almost no epidemiological studies have been conducted for Pineios river, thus it's microbial constitution is largely unknown. One of the few epidemiological studies had as an aim the study the levels of microbiological pollution in major Rivers in Northern Greece, a study that included Pineios River (Karanis et al., 2005). Although there has not been a significant epidemiological interest, due to the fact that the river crosses residential areas and is used as the main water source for the irrigation of Thessalian plain and thus the water is a lot of times tested for its microbial load, the interest of morphology, the quality and the quantity of water it provides is great. The consequences of geological and temporal changes due to climate change were studied through various experiments (Loukas, 2010; Panagopoulos et al., 2016). In addition, the recent floods of September 2023 changed temporarily but in a great manner the boundaries of the banks of the river. Epidemiological concerns were raised because of the mixing of the waters of the river with sewage septic animal products and various organic compounds of agricultural use. To our knowledge, to date, none of the published epidemiological studies included *Legionella spp.* or *Legionella pneumophila*.

In our experiments we conducted sampling of the Pineios river from early (05/06/2023) to middle summer (04/07/2023). The results displayed an increase of total bacteria with the rise of temperature (Figure 14). The optimal environmental temperature observed was at 34 ˚C. Total bacterial 16S rRNA marker gene displayed high positive correlation with the increase of temperature (r = 0.798). The occurrence of seasonal increase in river environmental ecosystems in Greece is a frequent phenomenon and the optimal ambient temperature range for microbial growth is 20-35°C (Anagnostopoulos et al., 2022; Cabral, 2010; Kritzberg & Bååth, 2022). This range could vary slightly depending on the specific species of bacteria that are concerned, but it is generally accepted that warmer temperatures tend to lead to higher microbial loads. Total bacterial counts increase along with temperature increase has been previously associated with heterotrophic bacterial proliferation at the range of temperatures observed in this study (Phillips et al., 2017). The results of *LEG* and the *Legionella* specific 5S rRNA gene marker exhibited a very high positive correlation (r = 0.842 and r = 0.734 respectively) with the rise of temperature, which is something expected as temperature has a significant role in the proliferation of *Legionella* spp. Growth of *Legionella* spp. has been demonstrated to be associated with both temperature increase when protist presence (protists act as reproductive hotspots for *Legionella*) is not restrictive (Caicedo et al., 2018). In eutrophication prone environments like that of Pineios river, protist presence is not expected to be restrictive. The results of the *L. pneumopila* specific *mip* marker gene showed almost no linear correlation (Correl= 0.096) with the rise of temperature. Previous studies with precision incubators testing temperature gradients have shown *L. pneumophila* optima at temperatures of approximately 42˚C, higher than

the ambient temperature of the Pineios river during the sampling period (Konishi et al., 2006). Hence, it is not surprising that no consistent changes with this gradient were observed.

While analysing the total prokaryotic diversity, we observed an increase in α-diversity with time/temperature. Although it is difficult to capture the dynamic nature of rivers through generalizations, α-diversity increase of planktonic bacteria has been observed with temperature (N. Liu et al., 2023). Such diversity increase may facilitate functional diversity and even functional redundancy, thus preserving microbial ecosystem services, with one of them being the control over putative pathogens like *L. pneumophila*. β-diversity analysis showed the increase of taxa like α-*Proteobacteria*, *Bacilli*, *Pseudomonadaceae*, *Acinetobacter* and *Chloroflexi*. All of these taxa are known to be or include microorganisms with tolerance or preference to increased temperatures (Asif et al., 2018; Bennett et al., 2020; Manaia & Moore, 2002; Singh et al., 2021).

Overall, it is important to understand distribution of *Legionella* in open waterbodies, within the context of the total microbial populations. Studies have shown that there is a positive correlation between the epidemiological surges of legionellosis under an environment under the influence of climate change (Han, 2021). Considering that *L. pneumophila* requires a parasitic intercellular relationship with the host for its proliferation and it is really dependent on its host. Climate change has effects in both of these above mentioned factors (Panagopoulos et al., 2016). Taking into account all the above, the dynamic environment of river Pineios requires constant monitoring for understanding the epidemiological significance of *L. Pneumophila*.

Almost no epidemiological studies have been conducted for Pineios river, thus it's microbial constitution is largely unknown. One of the few epidemiological studies had as an aim the study the levels of microbiological pollution in major Rivers in Northern Greece, a study that included Pineios River (Karanis et al., 2005). Although there has not been a significant epidemiological interest, due to the fact that the river crosses residential areas and is used as the main water sourcefor the irrigation of Thessalian plain and thus the water is a lot of times tested for its microbial load, the interest of morphology, the quality and the quantity of water it provides is great. The consequences of geological and temporal changes due to climate change were studied through various experiments (Loukas, 2010; Panagopoulos et al., 2016). In addition, the recent floods of September 2023 changed temporarily but in a great manner the boundaries of the banks of the river. Epidemiological concerns rised because of the mixing of the waters of the river with sewage septic animal products and various organic compounds of agricultural use. To our knowledge, none of the epidemiological studies included *Legionella spp.* or *Legionella pneumophila*.

In our experiments we conducted sampling of the Pineios river from early (05/06/2023) to midsummer (04/07/2023). The results displayed an increase of total bacteria with the rise of temperature. The optimal environmental temperature was at 34 ˚C. The occurrence of seasonal increase in river environmental ecosystems in Greece is a frequent phenomenon and the optimum temperature range of growth is 20-45°C. This range could vary slightly depending on the specific species of bacteria that are concerned, but it is generally accepted that warmer temperatures tend to lead to higher microbial loads (Anagnostopoulos et al., 2022; Cabral, 2010; Kritzberg & Bååth, 2022). The proliferation of *Legionella spp* and specifically *L. Pneumophila* displayed a similar pattern. Unfortunately due to the use of non-specific primers for analysis, time-limitations and most importantly the small number of samples and the limited period of sampling, definitive results about *L. Pneumophila*'s change in populations in accordance to temperature cannot be drawn. Studies have shown that there is a positive correlation between the epidemiological surges of legionellosis

under an environment under the influence of climate change (Han, 2021). On another light, considering that *L. Pneumophila* requires a parasitic intercellular relationship with the host for its proliferation and it is really dependent with the host's populations. The population of a microorganism is a multifactorial phenomenon that is not dependent just on the temperature but also the different necessary nutrients that the protists require, in addition to other factors. Climate change has effects in both of these above mentioned factors (Panagopoulos et al., 2016). Taking into account all the above, the dynamic environment of river Pineios requires constant monitoring for acquiring the data of epidemiological significance about *L. Pneumophila*.

5. Future Work

The current thesis revealed that more needs to be done in order to fully comprehend the ecology of *Legionella spp* and *L. pneumophila*, but also towards the study methodology of these microbial taxa. Within this context proposed future work includes the following:

- Expanding our sampling to a broader range of locations along the Pineios river and a broader scale of temperatures and timepoints. Moreover, the diversity analysis should include protists as well which serve as hosts facilitating the growth of *Legionella* spp.
- Performance of metagenomics analysis of the whole environmental DNA of Pineios samples and genome recruitment analysis against *Legionella* genomes. This could reveal the presence in tested samples of ''islands of pathogenicity'' that contain many important genes associated with the pathogenicity of *L. pneumophila*. Nevertheless, such approach makes difficult the confident connection of putatively horizontally transferred elements with their host of origin. According to studies, though, *Legionella pneumophila* is not considered a notorious carrier of a broad range of horizontally transferred elements commonly found in other bacteria.
- Population genomics approaches that can capture the genetic material enclosed in *Legionella* cells. They can further enlighten regarding the presence of mobile genetic elements (such as plasmids and transposons) and the traits that they confer to bacteria (regarding pathogenicity and antibiotic resistance).

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