Universidade do Minho
Escola de Engenharia

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Survival of drinking water pathogens after disinfection

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Survival of drinking water pathogens after disinfection

Dissertation for PhD degree in Chemical and Biological Engineering

Trabalho efectuado sob a orientação da
Professora Maria João Vieira e do
Professor Charles William Keevil

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Maria Salomé Gião Teixeira de Carvalho

“And if I have prophetic powers, and understand all mysteries and all science (…) but do not have love, I am nothing.”

I Corinthians 13:2

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"The value of things is not in how long they last, but in the intensity with which they happen. That's why there are unforgettable moments, unexplainable things and incomparable people"

(Fernando Pessoa)

No other words could sum up my PhD time any better.

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Abstract

Legionella pneumophila is a waterborne pathogen, ubiquitous in natural aquatic environments. It is also commonly found in engineered waters when disinfection fails and is responsible for outbreaks and sporadic cases of Legionnaires' disease worldwide. Conversely, the route of transmission of the gastric pathogen, *Helicobacter pylori,* is still unknown, but water has been recently considered a strong candidate for vehicle of transmission. A better knowledge on the survival strategies of these two pathogens to disinfection is therefore fundamental to achieve an efficient microbiological control of drinking water distribution systems (DWDS). These studies should however take into account that pipe surfaces of DWDS are colonized by heterotrophic populations of microorganisms that form structures denominated as biofilms, and that these biofilms might provide a protective haven for the survival of the pathogens studied here.

As such, the aim of this work was to study the effect of chorine on *L. pneumophila* and *H. pylori* cells, both in suspension and when associated with heterotrophic biofilms. The role of several physico-chemical parameters and of specific waterborne bacteria on the inclusion of these two pathogens into DWDS biofilms was also studied.

In the study of the influence of chlorine on *L. pneumophila* (Chapter 2), pure cells of *L. pneumophila* NCTC 12821 were suspended in tap water and different concentrations of chlorine were added to obtain final chlorine concentrations of 0.0, 0.2, 0.7 and 1.2 mg l^{-1}. Cells were then quantified by standard culture methods onto BCYE agar plates and by using a SYTO 9/Propidium Iodide-based viability kit. The cells exposed to 1.2 mg l^{-1} were also co-cultured with *Acanthamoeba polyphaga*. The results obtained showed that after exposure to low concentrations of chlorine, *L. pneumophila* can maintain viability even after a complete loss of cultivability, becoming viable but non-cultivable (VBNC). This condition was confirmed by the ability of *L. pneumophila* to recover cultivability after passage into amoebal cells.

In Chapter 3, the influence of several physico-chemical parameters on the inclusion of autochthonous *L. pneumophila* into heterotrophic drinking water biofilms was studied. The experiments were conducted in a two-stage chemostat system, with the second stage consisting of three vessels working in parallel at 20ºC. In a second experiment the temperature of the second-stage was decreased to 15ºC. The biofilm was formed on uPVC coupons and total cells, total and cultivable *L. pneumophila* and cultivable heterotrophic microrganisms were quantified. Cultivable *L. pneumophila* was never recovered from biofilms or the planktonic phase but results obtained using a peptide nucleic acid (PNA) probe showed that this pathogen will easily embed into potable water biofilms independent of the conditions tested. Temperature seems to be the parameter that most influences *L. pneumophila* numbers within DWDS biofilms, with a higher incidence being obtained at 15ºC.

To study the influence of low concentrations of chlorine on *L. pneumophila* associated to drinking water biofilms, chlorine was continuously fed to the second-stage chemostat to a final concentration of 0.2 and 1.2 mg l^{-1} (Chapter 4). The two concentrations of chlorine seem to have a little effect on the numbers of *L. pneumophila* cells, indicating that biofilms protect this pathogen from the oxidative stress of chlorine at concentrations higher than those commonly found in DWDS.

In Chapter 5 results obtained for *H. pylori* NCTC 11637 using a similar culture system and conditions described for Chapter 3 are presented. The inclusion of *H. pylori* in drinking water biofilms was not influenced by any of the conditions tested (temperature, shear stress or carbon addition). It was also observed that the shape of *H. pylori* cells is temperature dependent, being predominantly spiral at 20ºC and coccoid at 15ºC. The observation of *H. pylori* in biofilms after 31 days of inoculation demonstrates that biofilms are an important ecosystem in the protection of *H. pylori* under stress conditions.

The influence of chlorine on *H. pylori* cells both in suspension and associated to heterotrophic biofilms was studied (Chapter 6). The results showed that when in pure culture and suspension, *H. pylori* can completely lose cultivability without a significant loss of rRNA, possibly becoming VBNC. When associated to heterotrophic biofilms chlorine has also little effect on *H. pylori*.

In Chapter 7, the results obtained for the influence of several waterborne heterotrophic bacteria on the survival of *L. pneumophila* and *H. pylori* in dual-species biofilms are presented. The bacterium *Mycobacterium chelonae* appears to have a crucial role in the increase of cultivability of both pathogens, indicating that a wider screening of microorganisms commonly present in water might identify species that support the survival of these two pathogens in DWDS.

In the end of this work it is possible to conclude that disinfection by chlorine must be handled carefully as VBNC *L. pneumophila* and *H. pylori* might remain in suspension and associate with biofilms afterwards. In biofilms, cells are not only protected from residual chlorine but also in such a physiological condition that allows them to divide (*L. pneumophila*) or concentrate (*H. pylori*) within these structures. As a result, the release of biofilm to water due, for instance, to changing hydrodynamic conditions, might at times release an infectious dose of either pathogen, which should certainly be a subject of public health concern. Better ways to control water quality and the sloughing of biofilms are therefore needed.

Sumário

A *Legionella pneumophila* é uma bactéria patogénica que pode ser frequentemente isolada em meios aquáticos naturais. Logo, pode também ser encontrada em águas potável, como resultado de uma desinfecção deficiente, o que a torna responsável quer por casos esporádicos, quer por surtos de doença do Legionário em todo o mundo. Pelo contrário, a via de transmissão do patogénico gástrico *Helicobacter pylori* continua por desvendar, sendo no entanto a água um forte candidato. Por esta razão, é fundamental compreender que estratégias permitem a estes dois patogénicos sobreviverem ao processo de desinfecção dos sistemas de distribuição de água potável (SDAP), permitindo, assim, adoptar medidas que visem o controlo eficaz da qualidade desta água. Salienta-se, no entanto, que estes estudos apenas estão completos quando também se considera o importante papel dos biofilmes que se formam nas tubagens dos SDAP e que representam um refúgio para estes microrganismos.

O objectivo deste trabalho foi estudar o efeito do cloro livre em células em suspensão de culturas puras de *L. pneumophila* e *H. pylori*, bem como o efeito deste desinfectante nestes dois patogénicos quando associados a biofilmes heterotróficos. Foi ainda estudada a influência de diversos parâmetros físico-químicos e de determinadas bactérias isoladas de água potável na inclusão da *L. pneumophila* e da *H. pylori* em biofilmes.

No estudo da influência do cloro em células de *L. pneumophila,* (Capítulo 2) foram preparadas suspensões puras de *L. pneumophila* NCTC 12821 em água da torneira previamente filtrada e adicionadas diferentes concentrações de cloro (0.0, 0.2, 0.7 and 1.2 mg l^{-1}). A concentração de células foi avaliada por diferentes métodos que incluíram cultivo em placas de agar de BCYE e o uso de kit de viabilidade bacteriana SYTO 9/Iodeto de Propídio (PI). As células tratadas com 1.2 mg l^{-1} foram ainda co-cultivadas com *Acanthamoeba polyphaga*. Os resultados obtidos demonstraram que a exposição desta bactéria a baixas concentrações de cloro resultaram na perda de cultivabilidade sem no entanto ocorrer perda total de viabilidade, pelo que se pode concluir que estas células após contacto com este desinfectante entram no estado de viáveis mas não cultiváveis (VBNC) conseguindo recuperar a sua cultivabilidade após infectarem células de ameba.

No Capítulo 3 foi estudada a influência de diversos parâmetros físico-químicos na inclusão de *L. pneumophila* autóctone em biofilmes de água potável. Para tal, utilizou-se um sistema de quimiostatos, cuja segunda parte era constituída por 3 fermentadores que trabalhavam em paralelo e a 20ºC. Numa segunda experiência diminuiu-se a temperatura de operação para 15ºC. A formação de biofilme foi promovida na superfície de cupões de uPVC e posteriormente removido para a quantificação de células totais, do número de células de *L. pneumophila* cultivável e total e bactérias heterotróficas cultiváveis. A recuperação de células de *L. pneumophila* cultiváveis nunca foi possível, quer da fase em suspensão quer dos biofilmes, no entanto o uso de sonda de PNA demonstrou que este patogénico pode facilmente ser incorporado em biofilmes heterotróficos independentemente das

condições ambientais. Por outro lado, constatou-se uma maior incidência de *L. pneumophila* em biofilmes formados a 15ºC o que demonstra uma maior sensibilidade a variações térmicas.

Foi ainda estudada a influência de concentrações baixas de cloro em células de *L. pneumophila* associadas a biofilmes, tendo-se para tal alimentado continuamente dois dos reactores do segundo estado com cloro obtendo-se uma concentração final dentro de reactor de 0.2 e 1.2 mg l^{-1} (Capítulo 4). Ambas as concentrações de cloro parecem ter um efeito insignificante na concentração de *L. pneumophila* associada a biofilmes indicando que este tipo de ambiente funciona como um refúgio para este patogénico ao efeito oxidativo do cloro.

No Capítulo 5 utilizou-se o mesmo sistema descrito no Capítulo 3 tendo no entanto os fermentadores sido inoculados com *H. pylori* NCTC 11637. Verificou-se que nenhuma das condições estudadas (temperatura, tensão de corte ou aumento da concentração de carbono) influenciou significativamente a concentração de *H. pylori* dentro dos biofilmes. Contudo foi constatado que a forma fisiológica desta bactéria era predominantemente espiral a 20ºC enquanto que a 15ºC a maioria das células se apresentava sob a forma cocóide. Por outro lado, a recuperação do biofilme de *H. pylori* demonstra que estas estruturas representam um ecossistema importante que protege este patogénico de condições de stress.

Foi também estudada a influência do cloro em células de *H. pylori* em suspensão e quando associadas a biofilmes heterotróficos (Capítulo 6). Os resultados demonstraram que este patogénico é capaz de perder completamente a cultivabilidade retendo contudo a viabilidade, tornando-se VBNC. Verificou-se ainda que este desinfectante tem um efeito desprezável em *H. pylori* associado a biofilmes heterotróficos.

No Capítulo 7 são apresentados os resultados obtidos no estudo da influência de diversas bactérias isoladas de água potável na sobrevivência de *L. pneumophila* e *H. pylori* em biofilmes de duas espécies. Não foram obtidos todos os resultados pretendidos no entanto verificou-se que a bactéria *Mycobacterium chelonae* parece desempenhar um papel fundamental no aumento da cultivabilidade de ambos os patogénicos.

No final deste trabalho é possível concluir que a desinfecção através do uso de cloro deve ser cuidadosamente estudada, uma vez que *L. pneumophila* e *H. pylori* no estado de VBNC podem permanecer em suspensão e associarem-se posteriormente a biofilmes. Dentro destas estruturas, as células não estão apenas protegidas do cloro residual como também são capazes de se multiplicar (*L. pneumophila*) ou concentrar (*H. pylori*). Como resultado, o desprendimento destas células para o fluído, devido por exemplo, à mudança das condições hidrodinâmicas, pode originar o aparecimento de doses capazes de causar infecções o que será, definitavamente, um problema de saúde púbica. Deste modo, é real a necessidade de um melhor controlo da qualidade da água e do desprendimento de biofilmes das paredes das tubagens de SDAP.

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List of symbols and abbreviations

ASHRAE	American Society of Heating, Refrigerating and Air Conditioning Engineers
BCYE	Buffered charcoal yeast extract
C	Carbon
CBA	Columbia blood agar
CCAP	Culture collection of algae and protozoa
CDC	Centres for Disease Control and Prevention
CFU	Colony forming units
Cl_2	Chlorine
CO_2	Carbon Dioxide
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
cys	L-cysteine
DBP	Disinfectant by-product
DLVO	Derjaguin-Landau-Verwey-Overbeek
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPD	N,N-dimethyl-p-phenilenediamine
DWDS	Drinking water distribution system
EDIC/EF	Episcopic differential interference contrast/epifluorescence
EPS	Extracellular polymeric substances
EWGLI	European Working Group for *Legionella* Infections
FISH	Fluorescence *in situ* hybridization
G + C	Guanine + Cytosine
GI	Gastrointestinal
GVPC	Glycine, vancomycin, polymixin and cycloheximide
H_2	Hydrogen
HOCl	Hypochlorous acid
HP	*Helicobacter pylori* selective agar
HPC	Heterotrophic plate count
HSC	Health and Safety Commission
HS/LC	High shear/low carbon
HSE	Health and Safety Executive
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride
LS/HC	Low shear/low carbon
LS/LC	Low shear/high carbon
MALT	Mucosa associated lymphoid tissue
MDPE	Medium density polyethylene
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

N / N_2	Nitrogen (element / molecular)
NCTC	National Collection of Type Cultures
NHMRC	National Health and Medical Research Council
O_2	Oxygen
OCl^-	Hypochlorite ion
P	Phosphate
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PNA	peptide nucleic acid
PPG	Proteose peptone glucose
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SEM	Scanning electon micrograph
TEM	Transmission electron micrograph
TTC	2,3,5-triphenyltetrazolium chloride
UK	United Kingdom
uPVC	Unplasticized polyvinylchloride
US	United States
UV	Ultraviolet
VBNC	Viable but non cultivable
v/v	Volume/volume
WHO	World Health Organization
WTS	Water Treatment Station
w/v	Weight/volume
XTT	sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzenesulfonic acid hydrate

Chapter 1 Background and Aims

Water is crucial to the life of organisms from the simplest prokaryote to the most complex eukaryote. To make drinking water safe for consumption, water must pass through several treatments that can vary as a function of the raw water quality. Nevertheless, disinfection is mandatory and from all the disinfectants used, chlorine is the one used in most of the water treatment stations.

Disinfection does not kill all the microorganisms present in water and those that remain will tend to form biofilms. One of the biggest problems of drinking water biofilms is the role that they assume in the protection of pathogens that when released to water might cause infections. Several methods can be used to detect those pathogens but cultivable methods are the most commonly and routinely used. These methods fail, however, to detect viable but non-cultivable bacteria.

Legionella pneumophila is a waterborne pathogen that still causes several outbreaks of respiratory disease worldwide. On the other hand, the route of transmission of the gastric pathogen, *Helicobacter pylori*, is still unknown. *H. pylori* might use several pathways to infect a human host and water is one of the possible ways.

1.1 Is drinking water safe?

“The human right to drinking water is fundamental for life and health. Sufficient and safe drinking water is a precondition for the realization of all human rights.”

United Nations, 2002 [60]

In 2002, 1.1 billion of people did not have access to safe drinking water supplies, mainly in African and Asian countries, and 3900 children died every day from diseases caused by waterborne pathogens. This happens due to inefficient sanitation, and therefore these diseases rarely occur in developed countries [133, 296]. However, water that can be used as drinking water is not as safe as it could be (as will be show in section 1.1.2) and is not homogeneously distributed worldwide. Besides, population growth, the increase in industrialization, and general pollution and glaciers melting are contributing to the decrease of freshwater sources and leading to a potential water crisis (Figure 1.1) [279, 296].

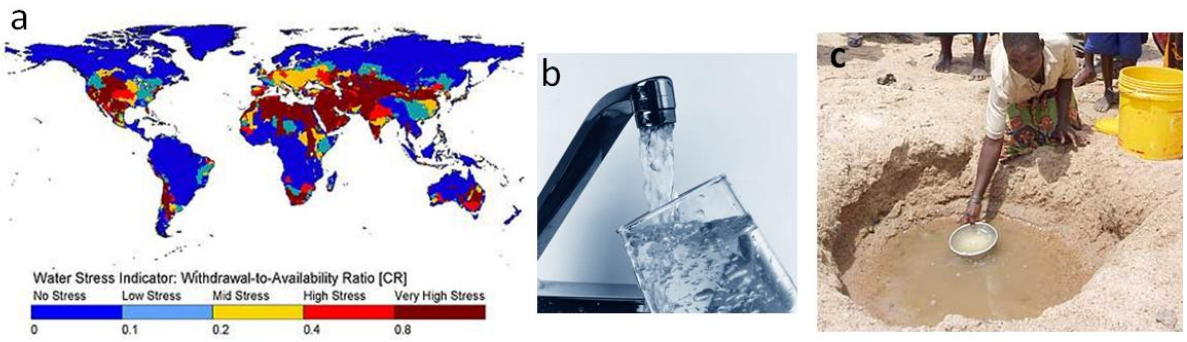


Figura 1.1 Water stress worldwide [296] (a) and differences in the assessment of drinking water in a developed [194] (b) and in a third world country [62] (c).

1.1.1 Brief history

Water is older than the Earth itself. Hydrogen (the oldest and most abundant element in the Universe) combined with Oxygen (formed in the womb of the stars) to form water (H_2O) even before the formation of the earth [17]. In fact, the first two letters of “earth” is the name “Ea” that means “house of water”. Ea-Enki was the name of a prehistoric Sumerian god as well, presented as coming from the sea and connected to the creation [164]. Since the appearance of humanity men have always been conscious about the importance of water in their lives and the first known settlements were close to abundant water sources (Table 1.1) [17, 216].

Table 1.1 Early civilizations and water basin associated.

Civilization	Date of first settlement	Water basin location	
Sumerians	6000 B.C.	Tigris-Euphrates valley (Mesopotamia)	
Egyptians	5000 B.C.	Nile valley	
Ancient Chinese	4500 B.C.	Hwang Ho valley	
Indus Valley	3000 B.C.	Indus Valley	

Source: [104, 139, 263, 273]

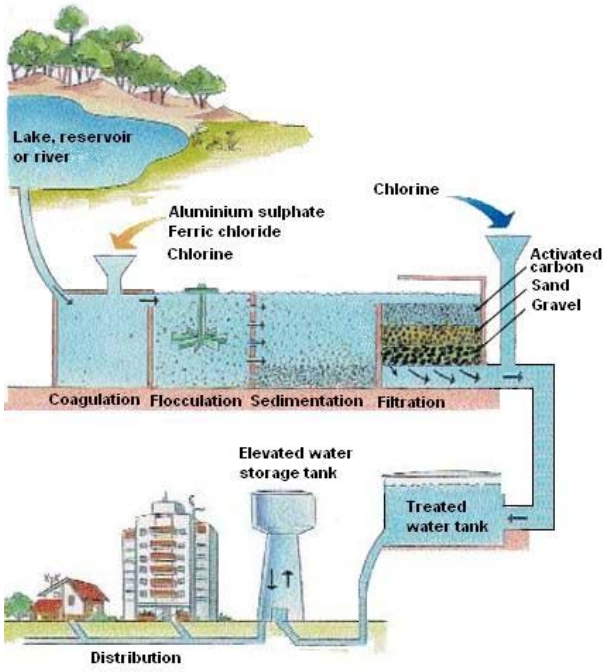
Water is probably the most precious commodity in life, not only in biological aspects but also from a social, economic, health, technical, financial and political point of view [262]. Across History it is possible to find a relation with water in several fields: religious rites, science, art, music, mythology, transportation, power, heating, architecture, etc. [17].

1.1.2 Water: from nature to tap

Water is fundamental in all biological processes independent of the complexity of the organism. Even the most primitive microorganism needs a minimal amount of water to perform its basic metabolic functions. Human beings can survive for almost 50 days without eating but no more than a few days without drinking [189, 245]. Around 70% of the earth surface is water (in a total of 1.4×10^9 km^3) but only 2.5% is freshwater. However, most of this water is trapped in glaciers and permafrost meaning that only 0.01% of the total water is available for consumption [188, 268].

The origin of drinking water can be superficial (including streams, rivers, lakes and dams) or groundwater (such as wells, springs and holes). Prior to A.D.1600, the consumption of water was based on visual clarity. If necessary, treatments such as exposure to sunlight, dipping of heated copper or other metals, boiling or filtration through a cloth were performed. In 1600 the treatments applied to water started to improve: water was therefore treated by the addition of germicidal metals (such as silver or copper), sand filtration, distillation, coagulation and adsorption with different materials. But it was only in the late 1800's that the first disinfectant was used [22, 283]. Table 1.2 summarizes the principal processes in modern water treatment stations (WTS).

Table 1.2 Current drinking water treatments.

Method	Difficulty	Cost	Pathogens removal	Diagram of a water station plant
Acids & bases	☺	$$	√√	
Adsorption	☹☹☹	$$$$	variable	
Aeration	☺	$	√	
Chloramines	☺☺	$$$	√√√	
Hypochlorite	☺☺	$$$	√√√√	
Chlorine gas	☹☹☹	$$$	√√√√	
Chlorine dioxide	☹☹☹	$$$$	√√√√	
Coagulation	☹☹☹	$$$	√√	
Filtration	☹☹☹	$$$$	√√√	
Ion exchange	☹☹☹☹	$$$$	√	
Ozone	☹☹☹☹	$$$$	√√√√	
Sedimentation	☺☺	$$	√	
Silver / copper	☺	$$	√	
UV lamps	☹☹☹	$$$	√√√	

Legend: ☺ to ☹☹☹☹: easier to more difficult;
$ to $$$$: cheaper to more expensive;
√ to √√√√: low to high removal
Source: adapted from [198, 283];

1.1.2.1 Coagulation

This process consists of the addition of chemicals to adjust the pH of the water and facilitate the following steps. Coagulants that destabilize and aggregate particles, forming suspended colloids, can also be added to the water. This treatment prepares the water for the next step, which is usually flocculation. The most commonly used coagulants are aluminium sulphate and ferric chloride [4, 6, 185]

1.1.2.2 Floculation

The destabilized aggregates may now collide and form heavier and larger particles – the flocks – that will settle out easily during the sedimentation process [4, 185].

1.1.2.3 Sedimentation

The velocity of water is decreased during its passage in the sedimentation basin to allow the suspended material and flocks to settle out on the bottom by gravity before the water exits the basin. The removal of some particles facilitates the filtration process avoiding the quick clogging of filters [5, 185].

1.1.2.4 Filtration

While groundwater is naturally filtered when passing through the porous layers of the soil, surface water has to be filtered in the WTS. This process is applied to remove organic particles (vegetation, humic substances), coagulation precipitates (such as precipitates of aluminium or iron), clay and silt particles, microorganisms and other suspended matter achieved by the passage of water through a filter (permeable fabric or a porous bed of materials) [7, 92, 185].

1.1.2.5 Disinfection

A common mistake is the confusion of disinfection with sterilization, as they are different. By definition sterilization is a process that eliminates all forms of life, including live microorganisms and spores, while disinfection is generally regarded as only killing live microorganisms but having no effect on spores [32]. In drinking water distribution systems (DWDS), disinfection is understood as a process that kills or inactivates microorganisms (especially pathogens) to a safe level, i.e., until a harmless concentration is reached [184, 283]. In DWDS disinfection can occur in two stages: primary (that aims to inactivate or kill microorganisms to a desired level) and secondary disinfection. In this latter step, it is necessary that a defined level of disinfectant remains in the treated water to prevent microbiological regrowth [184]. As such, the disinfection step is absolutely necessary to make water safe. However, some disinfectants can react with organic matter producing undesirable disinfectant by-products (DBP), some of them suspected to be carcinogenic and mutagenic. The type of DBP is dependant on the disinfectant used and the chemical composition of the water [35, 224, 243, 282].

Although chlorine is the universal disinfectant used in DWDS, there are other disinfectants that can also be used, depending on availability, difficulty, cost and efficacy [51, 117, 283]. Table 1.3 summarizes the most common disinfectants currently used in drinking water stations.

Table 1.3 Principal disinfectants used in DWDS and associated characteristics.

Disinfectant	Mode of action	Principal DBP	Microorg. effective	Residual disinf.
Ozone	As an oxidative, reacts with cytoplasmic substances leading to degradation of chromosomal DNA in bacteria and viruses and damaging protein coat of viruses	Carboxylic acid Aldehydes Ketones Di-carboxyl	Bacteria Viruses Cryptosporidium Giardia	No
Ultraviolet radiation	UV penetrates the microbial cell wall disrupting the genetic material, making reproduction impossible	No DBPs formed	Bacteria Viruses	No
Chlorine dioxide	Inactivates the phosphotransferase with consequent inhibition of the respiration metabolism. Can also react with viral RNA.	Halocetic acids Haloacetaldehydes Chlorates Toxic chlorites	Bacteria Viruses Cryptosporidium Giardia	Yes
Chloramines	Inactivates the energy-producing enzymes. Inactivates also the phosphotransferase inhibiting the respiration process.	Trihalomethanes Halocetic acids	Bacteria	Yes
Chlorine	The mode of action is not well known, but probably at low concentrations penetrates in the cell and reacts with enzymes and protoplasm while at higher conc. oxides the cell wall destroying the organism.	Trihalomethanes Hydroxyl radicals	Bacteria Viruses Cryptosporidium Giardia	Yes

Source: [35, 179, 184, 230, 283, 291, 299].

1.1.3 Chlorination of drinking water

Chlorine was the first disinfectant used to disinfect drinking water and has been used for more than 150 years (Figure 1.2a). The first time that chlorine was used as a disinfectant in water was in 1846 by Ignac Semmelweis in the Vienna General Hospital maternity unit to wash and disinfect hands before touching newborn children, and since then it has became widely used worldwide. The reason why chlorine is the most commonly used disinfectant is due to its effectiveness, easy of use and low cost. Furthermore, it can provide a residual disinfectant in water that prevents (or should prevent) the microorganisms' regrowth [92, 250, 283, 299].

Chlorine can be added to water in three different forms; chlorine gas, sodium hypochlorite and calcium hypochlorite. Once in water, chlorine first reacts with the organic compounds present in water forming secondary products that include DBP's and only after the breakpoint there will be chlorine available as a disinfectant (Figure 1.2b). Chlorine then reacts to form hypochlorous acid (HOCl) and hypochlorite ion (OCl^-) as described by the equations in Figure1.2c. These two species are known as free chlorine and both have oxidative power. However, as HOCl is neutrally charged, it penetrates the cell easier, being more effective than OCl^-. In fact, the effect of the hypochlorite ion as a disinfectant is so low that it is practically insignificant [149, 179]. The concentration of these two species in water is pH

dependent, as observed in Figure 1.2c. At pH 6 the concentration of hypochlorous acid is approximately 98% decreasing to 83% at pH7 and to 14.5% at pH 8.5, meaning that chlorination will be more effective when pH is between 6 and 7 [149].

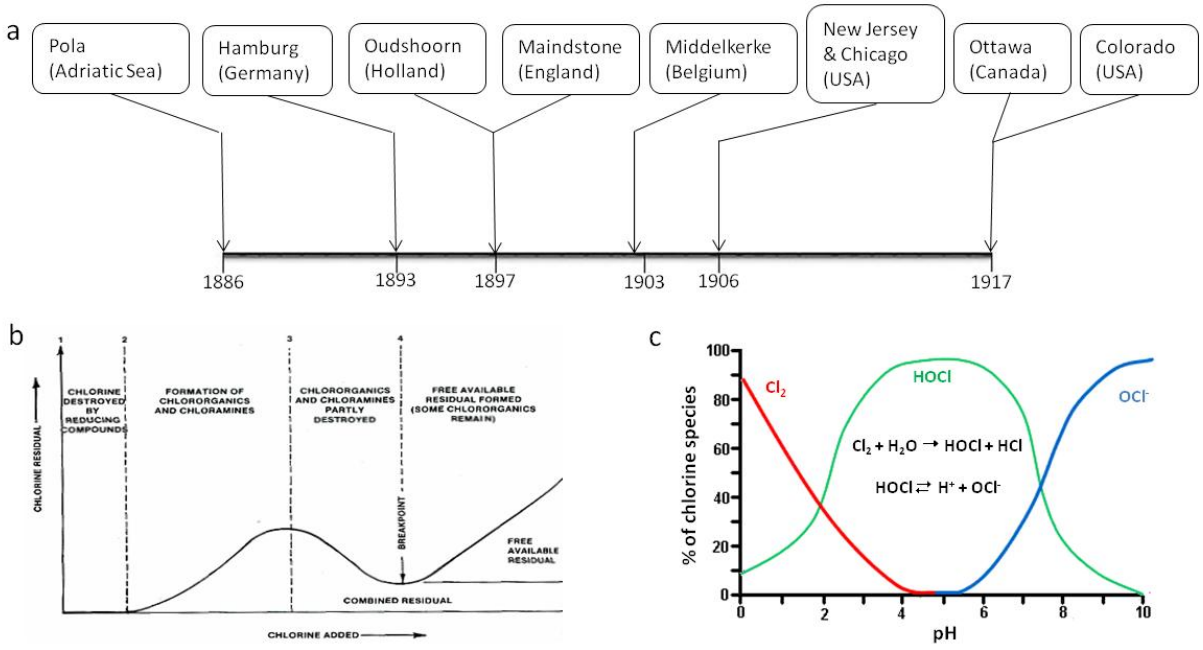


Figura 1.2 Chronology of drinking water chlorination [250, 283] (a). Drinking water chlorination: reaction of chlorine with organic compounds present in water and formation of free chlorine [181] (b). Chlorine reactions in water: formation of chlorine species dependant on water pH and respective chemical reactions [9] (c).

Investigations into drinking water chlorination are quite vast, from the characterization of DBPs and their effect on human health [27, 66, 74, 192, 298, 299, 303] to pathogen resistance. To give some examples, Mir and colleagues [176] have studied several Gram-positive and Gram-negative bacteria isolated from chlorinated water and concluded that Gram-positive strains are in general more resistant to chlorine. Lisle et al. [156] have demonstrated that *Escherichia coli* can survive higher concentrations than the residual chlorine concentration left in US water treatment stations. Pathogens such as *Clostridium perfringens* [208], *Mycobacterium* spp [149], *L. pneumophila* [146] and *H. pylori* [24] were found to be more resistant to chlorination than *E. coli*, the microorganism that is routinely tested as an indicator of faecal pollution for assessing and maintaining adequate water quality; consequently the role of *E. coli* has an effective indicator of treated water quality must now be questioned.

When inside the cell, chlorine may affect several components of the microorganism. Some authors have shown that hypochlorous acid is a multitarget reagent that can lead to damage to the DNA, cell walls, thiol and thiol groups, aminogroups in proteins with consequent cell inactivation or even death

[26, 70, 72, 97, 170, 173, 217, 241, 260]. Most of the studies concerning the deleterious effect of chlorine are conducted on the bacterium *E. coli*. As said before this microorganism is less resistant than some of the pathogens present in water and that, in conjunction with the fact the hypochlorous acid might affect cells in different ways, brings the necessity of further investigations into the effect of chlorination on other pathogens.

1.1.4 Waterborne pathogens

John Snow, an English physician from the XIX century, was the first to connect disease to water. It was during the outbreak of cholera in 1854 in Soho, London, that he proved that cholera was transmitted by water and not by air, as was the general opinion in the scientific community at the time. In his epidemiological studies he showed that most of the deaths occurred in the neighbourhood of the Broad Street (currently named Broadwick Street) water pump (Figure 1.3a) and for those that were living in other areas he managed to find out that they had drunk from the same water source [46, 203, 261]. However, it was only in 1884 that the microorganism responsible for this fatal disease was isolated by the German physician and researcher Robert Koch [144] and later called *Vibrio cholera* (Figure 1.3b).

A waterborne pathogen might be a bacterium, virus or protozoa that can cause disease and is transmitted by water, although it might also be transmitted by other routes, such as food, person-to-person contact or air [63, 85, 152, 272]. The introduction of these pathogens in water normally occurs by contamination with faecal matter, but some of them are ubiquitous in natural reservoirs [63, 85, 274]. For most waterborne pathogens water is a poor nutrient environment and these microorganisms have to adapt to survive in such stressful conditions until they reach a suitable host. Depending on the microorganism they might become viable but non cultivable (VBNC), associate with other microorganisms such as amoebal species, form a capsule, or attach to biofilms (Table 1.4).

Figura 1.3 Picture of the Broad Street water pump, in Soho, London, UK (a) and coloured transmission electron micrograph (TEM) bacteria responsible for cholera: *V. cholerae* [253] (b).

When collected from natural reservoirs, water passes through several treatments in WTS. The type and number of treatments are dependent on the raw water quality, but disinfection is an obligatory step. The complete elimination of drinking water microorganisms is an utopia, it can not be achieved and would not be beneficial. The problem arises only when the microorganisms that remain in water are pathogens that still retain infectious ability [195, 210]. To cause outbreaks each pathogen has a minimal infection dose and therefore the objective is to maintain the water pathogen concentration below such levels, avoiding the spread of diseases. Nevertheless, for technical and economical reasons, the control of drinking water safety still relies on the detection/enumeration of total coliforms and *E. coli*. As stated above, *E. coli* is known to be less resistant to chlorination than several waterborne pathogens. Furthermore, presence/absence of this surrogate is not always related to them, especially to those that are considered ubiquitous microorganisms [92, 115, 274, 286, 297].

Table 1.4 Some examples of waterborne pathogens, associated disease and survival time in water.

Microorganism	Disease	Introd. in water	Survival in water	Survival strategy
Bacteria				
Vibrio cholera	cholera	FM; U	1	B, IP, VBNC
Salmonella spp.	Typhoid fever, Gastroenteritis, Septicaemia	FM; U	2	IP, VBNC
Escherichia coli	Hemorrhagic colitis, Hemolytic uremic syndrome	FM	3	IP, VBNC
Campylobacter spp.	Gastroenteritis	FM;U	2	B, VBNC
Helicobacter pylori	Peptic ulcer, gastric cancer	FM	1	B, VBNC
Legionella pneumophila	Legionnaire's diesease, Pontiac fever	U	3	B, IP, VBNC
Mycobacterium avium	Infections on keleton, soft tissues, repiratory, gastrointestinal and genitourinary track	U		B, IP
Pseudomonas aeruginosa	Mastitis, otitis, infections on respiratory and urinary tack	U	1	B, IP
Viruses				
Hepatitis A	hepatitis	FM	3	A
Enteroviruses	myocarditis, poliomyelitis meningoencephalitis	FM	3	S, IP
Rotavirus	Gastroenteritis	FM	3	A
Adenovirus	Gastroenteritis, pneumonia, ureteritis	FM	3	A
Protozoa				
Giardia lambia	Giardisis	FM	2	C
Cryptosporidium parvum	Cryptosporidiosis	FM	3	B, oC
Acanthamoeba spp.	Encephalitis, keratitis, uveitis	U	3	B, C, S

Legend: B: biofilm; C: cysts; oC: oocysts; IP: inside protozoa; S: suspension; VBNC: viable but non cultivable; 1: less than one week; 2: between one week and one month; 3: more than one month.
Source: [82, 102, 152, 201, 228, 242, 297, 300].

One of the major problems in the control of drinking water safety is the ability of some pathogens to enter a viable but non-cultivable state (VBNC). The VBNC state was first described by the group of Rita Colwell back in 1982 [301] and since then more than 400 papers have been written about the VBNC state in several bacteria (reviewed by Oliver [199]). Under some circumstances, microorganisms can stop their DNA multiplication but maintain minimal metabolic activity. As a consequence, these microorganisms fail to grow on artificial media as they normally would do (they are not cultivable) but are still viable and able to recover, some by the simple addition of nutrients (such as *Salmonella enterica* serovar Enteritidis, *Pseudomonas fluorescens* and *Pasteurella piscicida*) others only by passage in other microorganisms. For instance, *L. pneumophila*, *M. avium* and *H. pylori* are able to be resuscitated after coculture with amoeba species. Other pathogens recover cultivability through passage in animals such as rabbits (*V. cholerae*, *E. coli*) or guinea pigs (*L. pneumophila*) [199, 200, 294, 301]. Additionally, it has already been proved that VBNC microorganisms maintain their pathogenicity and cause diseases after cultivability recovery [23, 94, 124, 132, 219]. The assessment of VBNC cells is therefore challenging and several techniques have been developed to attempt to detect pathogens that have entered this state as resuscitation in hosts is not practical for routine analysis. This will be discussed in the section 1.3.

1.2 Biofilms

In the natural environment, the physico-chemical conditions are usually adverse to microorganisms. Factors such as temperature, nutrient availability and oxygen concentration can cause stress in microbial cells. Microorganisms often do not live as free swimming (planktonic) cells but tend to adhere to surfaces. In these environments, microorganisms are surrounded by a matrix constituting of extracellular polymeric substances (EPS) and water. The community of microorganisms (sessile cells) embedded in the matrix is called a **biofilm** (Figure 1.4) [71, 129, 290]. Biofilms are well organized structures where bacteria are protected from environmental stress and can interact with other cells in antagonism, mutualism, competition and synergy relationships [44, 56, 190, 225, 258]. The way that cells communicate and organize in a social community is controlled by the secretion of signal molecules in a process called “quorum sensing”. The secretion of these signal molecules (called autoinducers or quormon) promotes the communication between cells and regulates the relationship between bacteria resulting in a group behaviour instead of an individual performance, e.g. cells can have a different function depending of their location in the biofilm [67, 205, 290].

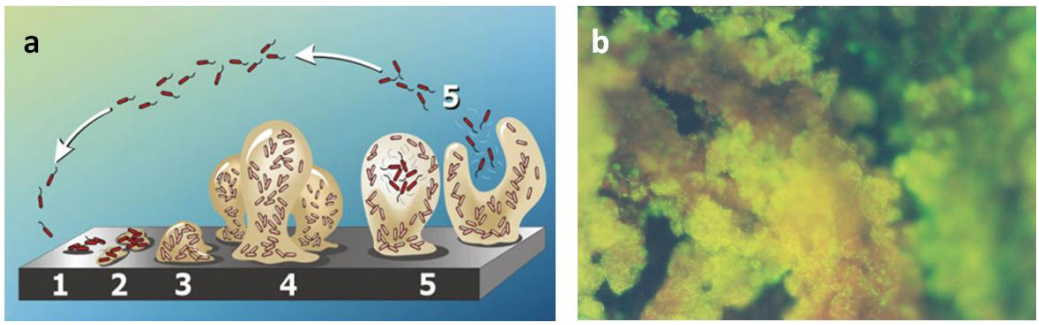


Figura 1.4 Stages of biofilm formation showing structure and architecture: 1: Initial attachment; 2: Production of EPS; 3 & 4: Maturation; 5: Dispersion of single cells [270] (a). Microphotograph of a *Pseudomonas fluorescens* biofilm where the EPS is stained with Live/Dead® *Bac*Light™ kit [96] (b).

Research into the study of biofilms has been ongoing for over 70 years. The first observations that bacteria prefer to live in biofilms instead of living a the planktonic state dates back to 1933 [116] and the first laboratory studies were conducted some years later by Zobell [304, 305]. In the last 30 years there has been an explosion in the amount of research into biofilms.

1.2.1 Formation of biofilms

Biofilms formation occurs in four main stages: transport of the microorganisms to the surface, initial adhesion, maturation and detachment (Figure 1.4a).

The first step of the formation of biofilm is the transport of the microbial cells to the substratum surface. The transport can be due to Brownian motion, transport through the boundary layers, motility by cellular locomotive structures such as flagella and, in quiescent conditions, sedimentation [93, 223].

When the cells approach the surface they can interact with each other by the establishment of long and short/intermediate distance forces. The long distance forces are described by the Derjaguin-Landau-Verwey-Overbeek theory (DLVO forces) and comprise the attractive forces of van der Waals and the repulsive forces of the electrostatic double-layer. In equilibrium, when favourable, this results in the adhesion of microorganisms. The short/intermediate distance forces include hydrophobic interactions, hydrophobic pressure, steric forces, Born repulsion forces and polymer bridges [88, 93, 197].

The adhesion of specific microorganisms can be facilitated by co-aggregation (adhesion of suspended cells) and co-adhesion (between adhered and suspended cells). The co-aggregation is particularly important in the formation of biofilms in dental plaques and aquatic environments. The influence of co-aggregation in biofilms formed in dental plaque is well documented; however, this phenomenon is not well understood in the formation of aquatic biofilms [45, 140, 231].

After adhering to the substratum cells can grow and replicate. In the maturation phase, the development of a complex architecture with the formation of channels, pores and redistribution of bacteria along the biofilm is observed (clusters – Figure 1.4). It is in this stage that microorganisms produce large amounts of EPS that embed round the biofilm and protect the microorganisms inside from stress factors, such as the presence of biocides [42, 71, 196, 269].

The last phase is the detachment of cells and other components from the biofilm. The detachment occurs due to different mechanisms: grazing (predation by protozoa species), erosion (removal of small particles due to the shear stress of the fluid), abrasion (caused by collision and/or rubbing of particles that may be covered with biofilm) and sloughing (detachment of large portions of biofilm). When cells detach from the biofilm they might return to their planktonic growth and cause infections [38, 105, 232].

The maturation and detachment stages can occur simultaneously and the biofilm enters a dynamic equilibrium. For this reason older biofilms have a relatively constant biomass [42, 285].

1.2.2 Types of biofilms and their impact on public health

Biofilms can be formed by a single microbial species, such as some medical biofilms, or, more frequently, constitute a consortium of microorganisms which can include bacteria, viruses, fungi, algae and protozoa. The diversity of nutrients that can penetrate inside the biofilm will determine the heterogeneity of the biofilm [140, 196].

Depending on the situation, biofilms can be beneficial or detrimental. Biofilms used in pharmaceutical and fermentation industries, wastewater treatment stations and natural biofilms in lakes or rivers which contribute to pollutant degradation are considered beneficial, while biofilms that accumulate on heat exchangers, membrane systems, filters, drinking water pipes, catheters, medical implants, live tissues and contact lenses are detrimental (Figure 1.5) [53, 84, 172, 202, 206, 246].

Biofilms are quite often also associated to corrosion problems either by the production of elements that attack the material where they are formed (such as acids, minerals, ammonia) or by direct feeding on the material [22].

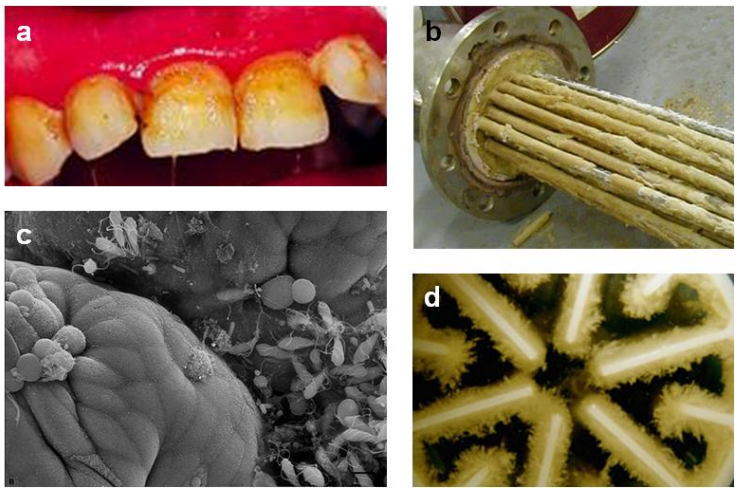


Figura 1.5 Photographs of different types of biofilms: dental plaque [271] (a), heat exchanger [125] (b), on gut tissues [249] (c) wastewater treatment [15] (d).

One of the biggest problems of biofilms as a form of life is their potential impact on human health. The susceptibility of sessile cells to disinfectants, biocides and antibiotics is much lower (can be up to 1000 times more resistant) than planktonic cells. Several studies have revealed that the penetration and diffusion of these products inside the biofilm matrix is very difficult and therefore the concentration of antimicrobial products needed is considerably higher and sometimes impracticable [122]. Furthermore, when sessile cells release from biofilms they can be able to return to their planktonic phase as infectious agents (in the case of pathogens). The fact that biofilm microorganisms are exposed to a sublethal concentration leads to the occurrence of resistant cells after returning to the planktonic phase, which in turn are more difficult to kill. The physiological mechanisms as to why cells become resistant are still unclear but in the last few years some theories have arisen to explain the increase of resistance in those cells which include horizontal gene transfer, the presence of altruistic cells and change in the cell phenotype [90, 105, 129, 145, 150, 151, 154, 206].

1.2.3 Factors affecting biofilm formation

Biofilms as live communities can be affected by several parameters, ranging from the type of microorganisms that constitute the biofilm to the physico-chemical parameters such as those stated below:

- **Temperature:** All microorganisms have an optimal growth temperature and variations of temperature will influence their development [226]. Consequently microbial adhesion and biofilm growth are temperature dependent [54, 159, 237].

- **pH:** A different pH from the optimal affects microbial metabolism as well as the superficial electric properties of the membrane interfering in the repulsion and attraction forces between the bacteria inside of the biofilm [212].

- **Shear stress:** Biofilms grown under turbulent flows are more compact which hinders the diffusion of nutrients and oxygen. However more turbulent flows leads to thinner biofilms and might force mass transfer, therefore increasing shear stress can be beneficial for biofilm formation [75, 121, 213, 277].

- **Presence of biocidal agents:** In DWDS, the biocide present by default, is chlorine. The presence of residual chlorine is one of the stress factors that leads to biofilm formation however, some studies have demonstrated that chlorine is also able to control biofilm formation by reducing the rate of biofilm growth, promoting biofilm detachment and decreasing the activity of microorganisms [58, 59, 68, 159].

- **Nutrients quantity and quality:** The presence of carbon compounds is essential to the formation of biofilm and the concentration of nutrients will influence metabolism with emphasis on EPS production. Other elements necessary for metabolism include mineral ions, phosphorous and nitrogen [33, 52, 59, 138, 186].

- **Microorganisms:** The type of microbial consortium present in the bulk water will determine the type of biofilm. Nevertheless, some studies have demonstrated that for some microorganisms in pure culture, the concentration in the bulk water do not significantly affect the concentration of the microorganisms in the biofilm [20, 50, 292].

- **Presence of particles:** Depending on the type of particles the effect will be two-fold. There are particles, like sand, that will promote the erosion of biofilm while others, like kaolin result in thicker and stronger biofilms [174, 212, 284].

- **Support material:** The influence of surfaces on biofilm formation is well documented for different microorganisms. Different materials support different biofilm formation but this is also dependent on the type of microorganism [18, 141, 178, 236].

However, in real life all these parameters work together to influence biofilm formation. In this way, the impact of some of them may be insignificant when compared with the impact of others and must therefore be considered carefully.

1.2.4 Drinking water biofilms

One type of biofilms which has been extensively studied is the biofilm associated with drinking water systems. The biofilms formed in DWDS have several implications, from aesthetic to public health concern and are influenced by several factors.

1.2.4.1 After disinfection why are biofilms a concern in DWDS?

As described previously, disinfection is not sterilization and after this drinking water treatment some microorganisms still remain in the water. From the disinfection point water has to pass through many kilometers of pipes until it arrives to the houses. Factors such as nutrient concentration, residual disinfectant and temperature cause stress in these microorganisms leading to attachment on the pipe walls and consequent biofilm formation. When cells detach from biofilms they can cause several problems that will be discussed below and when these microorganisms are pathogenic they might be related to the occurrence of outbreaks of disease [78, 105].

1.2.4.2 Which problems can drinking water biofilms cause?

The presence of biofilms in DWDS possibly will lead to three main problems: increase in water company costs, aesthetic inconveniences (taste and odour) and public health concerns. The first problem is connected to a decrease in water quality or obtaining false results following coliform tests which require strategies to provide safe drink water while aesthetic problems are related to the emergence of bad taste, odour, colour and presence of invertebrates in drinking water [22, 140].

When pathogens survive the disinfection stage they will remain in the water, incorporate into biofilms and survive for long periods and under adverse conditions. Whether they pose a public heath threat when re-entering the water and reverting to their planktonic phase is a subject of discussion. Payment et al. [209] did not find any relationship between biofilm presence in DWDS and occurrence of disease. However it has been proved that pathogens such as *L. pneumophila*, *Mycobacterium* spp., *Pseudomonas aeruginosa*, *Klebsiella* spp., and *Cryptosporidium*, are transmitted by contaminated water and biofilms are a good candidate as they can act as a protective niche to their survival in drinking water as previously showed by several authors (reviewed in [85, 256, 272]).

1.2.4.3 Pipe material: a dual problem

The water distribution network includes pipes of different materials with the most common being cast iron, medium density polyethylene (MDPE) and unplasticized polyvinylchloride (uPVC). All these materials can support biofilm formation although cast iron is preferred over the other two materials. Cast iron pipes provide iron, which is an essential element to the metabolism of most microorganisms. The presence of biofilms on cast iron surfaces results in the corrosion of these pipes (Figure 1.6) and loss of pipe material, a deleterious condition that has not been found when MDPE or uPVC are used. For these reasons in several countries when metallic water pipes need to be replaced they have used either MDPE or uPVC [22, 141, 193].

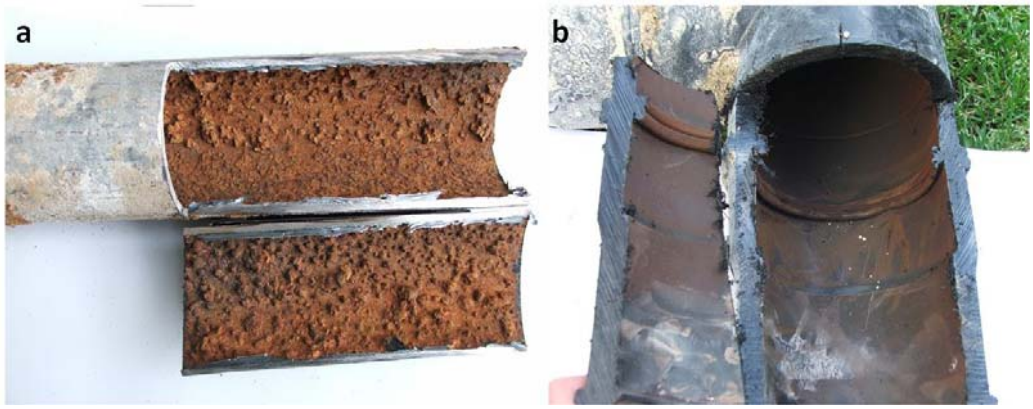


Figura 1.6 Photograph of a cast iron pipe where corrosion has occurred (a) and of a high density polyethylene pipe with biofilm (b) (photographs kindly yielded by Sofia Bragança).

1.2.4.4 Drinking water biofilms control

The formation of biofilms can be affected by several parameters that can be manipulated to control biofilm growth on DWDS. One of the strategies which can be adopted to control and remove biofilms from these systems is the increase of residual disinfectants such as chlorine. However, this has to be carefully studied as required levels could be too high to be practicable, due the introduction of strong odour and taste in the water, the increase of DBPs and the selection of resistant bacteria. The use of alternative disinfectants needs more research both with regards to the penetration into biofilms and consequent antimicrobial effect, their reaction with pipe material, especially cast iron, and the formation of associated DBPs. The control of carbon content in water is another strategy. In general, microorganisms need a C:N:P (carbon, nitrogen and phosphorous) ratio of 100:10:1 where carbon is the growth limiting nutrient, thus restricting the carbon concentration will decrease the microbial growth [52, 211]. This is, in general, reached by the decrease of organic matter content, however, it would be a very expensive process and ineffective for bacteria able to grow in oligotrophic environments. This last strategy relies on the replacement of the pipes with materials such as uPVC and MDPE that support less microbial adhesion than cast iron and on the other hand are less susceptive to residual disinfectant attack [140].

1.3 Assessment of sessile and planktonic microorganisms

As described in section 1.1.4, assessment of microbiological drinking water safety is performed by evaluation of the presence of coliform bacteria. To be considered safe no coliform bacteria should be detected in 100 ml of sample using standard culture methods [297]. However this method is not reliable for several reasons; VBNC cells may not be detected by cultivable methods, due the presence of microorganisms which are more resistant to chlorination and because of the existence of species which are ubiquitous in the environment (the detection of coliform bacteria assesses the contamination of water by faecal matter). In this section, methods that are currently used in research associated with drinking water pathogens and not necessarily used by drinking water treatment companies to routinely analyze drinking water, will be described.

1.3.1 Culture methods

Robert Koch was the first scientist to use the heterotrophic plate count (HPC) method to quantify heterotrophic microbial species in drinking water as a means of assessing drinking water safety [144]. Most of the HPC present in drinking water are innocuous to man, but some of them can be pathogenic especially to immunocompromised people; for example, some species of *Pseudomonas* and *Acinetobacter*. Additionally, the presence of pathogens that are non HPC, such as *Legionella* and *Mycobacterium* spp., has also been reported [98]. For simplicity and economic reasons, HPC are sometimes used in drinking water treatment stations to obtain a general idea of the water quality but it is not possible to distinguish/identify the bacteria isolated [211, 233]. In particular cases, selective media can be used to determine the presence of specific pathogens of interest. It should be noted that no medium is 100% specific for a particular microorganism and overgrowth of other species might be observed, so confirmative tests are needed afterwards [13, 108, 109, 110, 111, 112]. In Table 1.5 some examples of media (some commercially available) are shown which are used to recover specific bacteria.

The cultivable methods are primarily used to detect live microorganisms, however since the discovery of VBNC cells the state of art has changed. The use of cultivable methods to assess drinking water safety has to be handled carefully, because several pathogens are difficult to recover when in a VBNC state but they may be able to maintain their virulence and consequently cause infections. Several authors have already alerted the fact that cultivable methods should be, therefore, used with precaution and supported by other methods [239].

Table 1.5 Media used in drinking water analysis to recover specific microorganisms.

Microorganism	Media	Incubation
HPC	R2A; m-HPC agar; Plate count agar	22ºC – 28ºC for 5 – 7 days 35ºC for 48 hours
Salmonella	Brilliant green agar; Bysmuth sulfite agar; Xylose lysine desoxychocolate; MacConkey agar	35º – 37ºCfor 24±3 hours
E. coli	MacConkey agar Nutrient agar	30±1ºC for 4±1 hours followed 44±0.5ºC for 14±1 hours
Campylobacter	Columbia blood agar Campylobacter selective agar	80% N_2, 10% H_2; 10% CO_2 41,5ºC for 44±4 hours
Legionella	BCYE GVPC	36±1ºC for 10 days (observe 2 times in between)
P. aeruginosa	Pseudomonas agar + CN supplement	37 ºC for 22±2 hours + 22±2 hours

Source: [13, 108, 109, 110, 111, 112]

1.3.2 Microscopy methods

The first observation of bacterial and protozoa cells under the microscopy was achieved by Anton van Leeuwenhoek in the XVII century using a very simple microscope constituted by a small but powerful lens [251]. Microscopy techniques are now highly advanced and are routinely used in many research laboratories.

The emergence of staining dyes revolutionized microscopy methods. In the case of fluorescent molecules, they absorb light when an electron is excited by light. When the electron returns to the initial energetic level (which has lower energy), it emits a photon of light in a mechanism known as fluorescence. Fluorochromes are molecules which absorb light of a certain wavelength and emit light at another wavelength. Microscopes adapted with filters of different wavelengths allow the visualization of this fluorescence. According to the dye used, it is possible to assess the physiology of cells (Figure 1.7), although there is still some controversy in the scientific community about the validation of results, either in the efficient use of these dyes in each microorganism or the veracity of the relationship to physiological state. Numerous studies have already shown that they can be successfully used as an alternative to cultivable methods due to their reliability and rapid nature [36, 134].

Each stain has a characteristic wavelength for excitation and emission that results in the fluorescence of different colours and allows the use of different stains simultaneously. When stains have a low molecular weight they easily penetrate inside cells (in any physiological condition) and bind to nucleic acids. This is the case for 4',6-diamidino-2-phenylindole (DAPI), acridine orange, SYTO 9, etc which are used to quantify total cells. Alternatively, stains such as propidium iodide (PI), with a higher

molecular weight only penetrate cells with an injured membrane and are therefore an assessment of dead cells. A commercial combination of these two kinds of stains is the *Bac*Light™, which has been widely used to assess viable/dead cells in one step (SYTO 9/PI). However, it is important to note that dead cells in specific cases might have an intact membrane leading to false results. Before routine usage, these methods should always be validated by the use of other methods [36, 134, 157, 167].

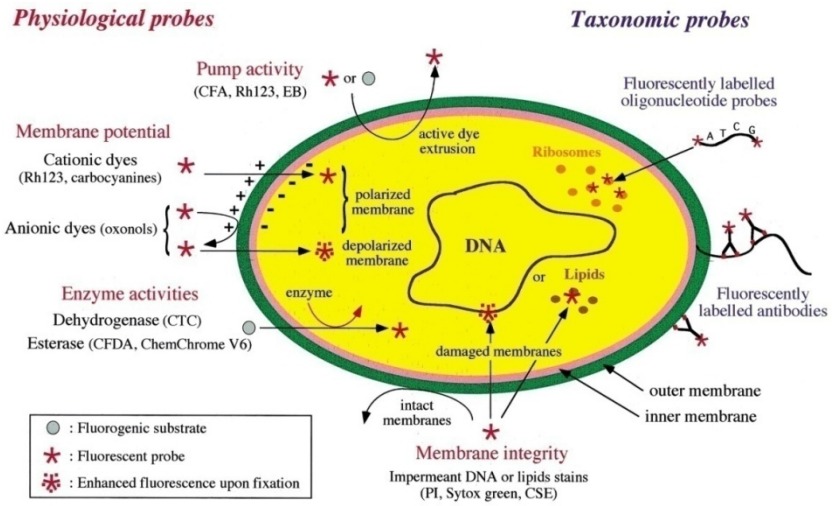


Figura 1.7 Diagram of the cellular targets of some fluorescent dyes [134].

Another way to determine the physiological state of a cell is by measuring repiratory activities by the use of different tetrazolium salts. Tetrazolium salts can be chemically or biologically reduced to an insoluble coloured formazan. Redox dyes include 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT). INT produces an opaque red formazan which is possible to observe using a bright-field microscope while CTC produces a fluorescent red formazan easier to visualize using fluorescence microscopy [235, 247]. CTC has been used to detect respiratory activity especially in aquatic bacteria and is suitable for VBNC detection [30, 229]. However, in some cases its use is difficult as metabolism may need to be stimulated by the addition of nutrients (such as carbon) and/or by changing the incubation parameters such as temperature or pH [41, 220]. In addition, other studies have demonstrated that CTC may be toxic for some species [255, 278].

Direct viable count is another method which has been used to identify viable cells, using nalidixic acid combined with a membrane-permeable stain as described above. Nalidixic acid inhibits DNA synthesis and consequent cell division but will not interfere with the metabolic state of the cell. The result is the appearance of elongated cells that correspond to viable cells that in some cases are not cultivable anymore. The elongated cells are visualized under microscopy after staining [41, 171, 259]. Another DNA inhibitor that is used to promote cell elongation is pipemidic acid [135].

1.3.3 Colorimetric methods

Colorimetric methods rely on the same principles of action as CTC but it is not observed microscopically. The reduction of nitroblue tetrazolium dyes by the radical superoxide (frequently present in biological systems) results in the formation of a soluble blue diformazan which can be measured spectrophotometrically at an appropriate wavelength. It is faster method of analysis than microscopic methods but less sensitive. The most common dyes include sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT), 2,3,5-triphenyltetrazolium chloride (TTC) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [41, 91, 238].

1.3.4 Flow cytometry

It is possible to measure the dispersion of light by microbial cells using a flow cytometer and correlate that dispersion to the number of cells present in the sample. This method is quite accurate but very complex as several experimental parameters need to be carefully chosen. Combined with fluorescent stains it gives extra information about cell physiology [218, 252].

1.3.5 Immunological methods

The principle of this technique relies in the fact that antibodies recognize three-dimensional epitope structures on specific microorganisms. Consequently, specific microorganisms can be detected and quantified in mixed samples [248, 275].

1.3.6 Molecular methods

Polymerase Chain Reaction (PCR) is a technique widely used to detect specific microorganisms by the targeting of specific genes and subsequent DNA amplification. One of the major disadvantages is the impossibility to distinguish viable from non viable cells and therefore its use in drinking water control should be limited as providing an indication of the presence/absence of a microorganism [302]. Quantitative PCR, that is able to distinguish DNA from viable and non viable cells by the use of ethidium monoazide and propidium monoazide has been recently under development. However it was not possible to successfully apply this technique to complex environmental samples [288].

In recent years, fluorescence *in situ* hybridization (FISH) has become a very popular method to detect and quantify specific pathogens in mixed consortia [128, 163].

The FISH method consists of the use of an oligonucleotide probe with a specific sequence that will bind to a complementary sequence on the DNA and RNA molecules. The sequence used determines whether the probe is specific for a whole genus or for a specific strain. The probes have a fluorophore

associated that will emit a signal when visualized under a UV light of a determined wavelength. It is always necessary to fix the cells (so they will not be removed in the subsequent steps and also to avoid the cell components being washed out from the cell), permeabilize the sample (to allow the probe to penetrate inside the cell), hybridize (where the probe penetrates and binds to the nucleic acids), wash (to remove non hybridized probe) and visualization. The use of probes with different fluorophores permits the detection of different microorganisms in the same sample [11, 12]. The first and most common probes are DNA-based, but in recent years, peptide nucleic acid (PNA) probes have appeared as easier and faster to use. The difference between these two probes is in the backbone: while DNA probes have a sugar phosphate backbone that is negatively charged, PNA has a polyamide backbone that is neutrally charged (Figure 1.8a). In this way, when PNA probes are used the hybridization is performed at low salt concentrations (improving the access to the target sequences by the destabilization of rRNA secondary structures) and avoids electrostatic repulsion. Furthermore, the hydrophobic nature of PNA facilitates the diffusion of the probe through the membrane and the synthetic nature of this molecule implies that it is not so susceptible to the attack of proteases and endonucleases [69, 191, 267].

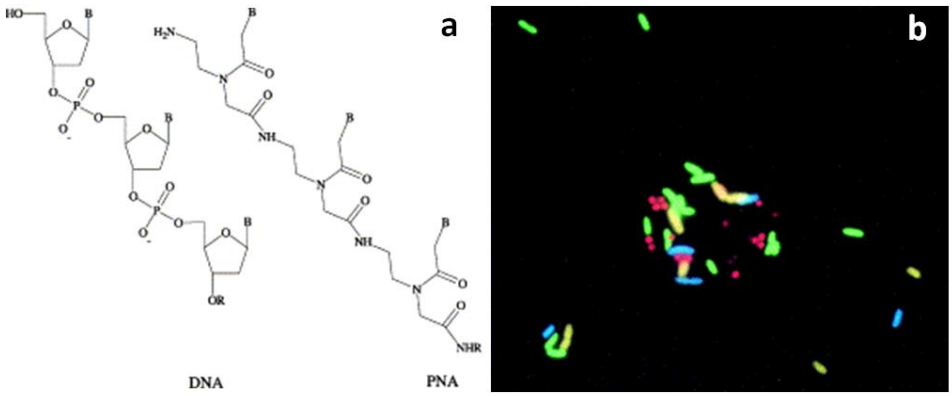


Figura 1.8 Structure of the DNA and PNA molecule (a) and microphotograph of a multiplex assay specific PNA probe for each strain (b) [215].

The use of PNA probes to detect specific pathogens in drinking water biofilms is gaining popularity, and although there is still some scepticism in the scientific community about the physiological information that PNA probes provide, several studies indicate that there is a relationship between the cells detected by PNA probes and viability [21, 153, 293].

1.4 *Legionella pneumophila*

In the last 30 years, much research has been done on the pathogen *L. pneumophila*, which remains responsible for deaths worldwide. Although nowadays the research is more focused on the clinical aspects, ecological aspects are still important, mainly because this microorganism is ubiquitous in natural environments and can survive the disinfection process.

1.4.1 Since the first outbreak

In 1976 during the 58th American Legion's convention hosted in Philadelphia, USA, more than 220 people fell ill and of those 35 people died of a mysterious disease. As the outbreak occurred during a Legionnaires' meeting and most of the affected people were Legionnaires or connected to them, the, then unknown, disease became known as "Legionnaires' disease". The first attempt to isolate the microorganism responsible for the outbreak failed and ironically it was thought that it was unlikely to be due to a bacterial infection. Only a few months later, early in 1977, Joseph McDade, using guinea pigs instead of mice isolated the bacterium that caused the outbreak during the previous August. The bacterium was named "*Legionella*" due to its connection with Legionnaires and "*pneumophila*" as it caused a pneumonic disease [87, 295].

This was the first *Legionella* outbreak reported although it is possible that several cases had occurred previously. In fact, McDade and colleagues identified a bacterium isolated in a pneumonia episode in 1947 as *L. pneumophila* [168]. Various outbreaks and sporadic cases have been reported and despite the fact that this disease is treatable, there are still deaths occurring worldwide due to this bacterium. Last year, 441 cases of Legionnaires' disease were reported in England and Wales of which 53 resulted in death [107]. In the United States, it is estimated that 8000 to 18000 cases of Legionnaires' disease occur every year [48]. However it is possible that these numbers are underestimated as in many cases the causes of pneumonal illnesss are not identified.

1.4.2 Characteristics

The family *Legionellacea* consist of one single genus named *Legionella* and are a subgroup of the γ-proteobacteria class. The nearest relative of the *Legionellacea* is the pathogen *Coxiella burnettii*, which causes a similar disease to legionellosis. The genus *Legionella* comprises 50 species, most of them pathogenic to humans, and 73 serogroups. *L. pneumophila* is the strain with most serogroups (16 in total and all of them pathogenic) and alone it is responsible for 98% of Legionnaires' disease, from which 95% are due to serogroup 1 [8, 158]. The complete genome of *L. pneumophila* is formed of 3.5 Megabase pairs, with a G+C content of 38% and approximately 3000 genes. On average 20% of the genes are unique to the genus *Legionella* [8, 55]. The *mip* genes are associated to their ability to infect and grow inside of phagocytic protist host cells, such as amoebae, and mammalian

phagocytes in the immune system, such as human macrophages. Additionally, the *icm* genes are associated with virulence of *L. pneumophila* [8, 57, 254].

L. pneumophila is a Gram-negative rod shaped bacterium (Figure 1.9c) of approximately 0.3 to 0.9 μm width by 2 to >20 μm length [295]. It has an aerobic and heterotrophic metabolism, and requires amino acids as source of carbon and energy as it is unable to oxidize or ferment carbohydrates [266]. In fact, *L. pneumophila* is not able to grow in artificial media in the absence of L-cysteine (unless when associated with other microorganisms such as *Flavobacterium breve*) [287] and requires a certain concentration of iron [127]. It does not form spores and can have pili and a flagellum. The optimal growth temperature is 35ºC, but it also grows well between 25º and 42ºC [83]. Nevertheless, it has been demonstrated that, in specific conditions, this bacteria can grow optimally at different temperatures, such as 30º or 45ºC [147].

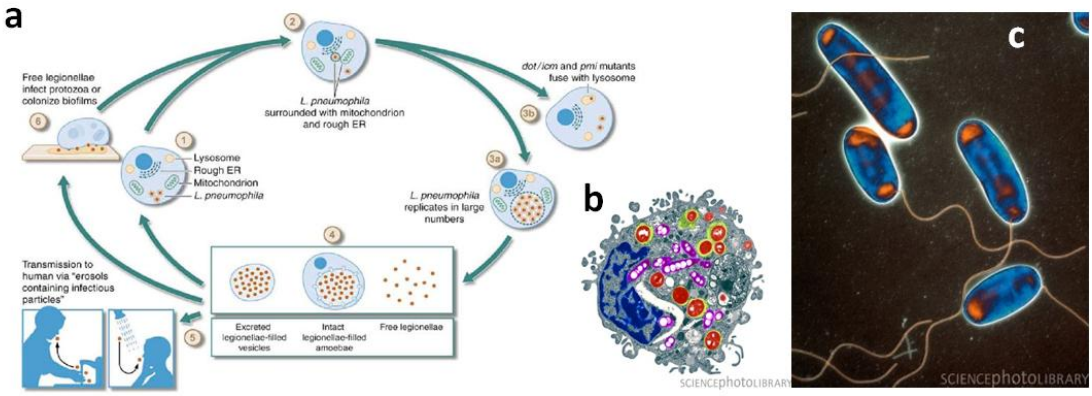


Figura 1.9 Diagram of the *L. pneumophila* cycle of life inside protozoa: 1. Environmental *L. pneumophila* in biofilms or infecting protozoa; 2 & 3. *L. pneumophila* inside of amoeba; 4. Infectous particles; 5. Transmission to humans; 6: *L. pneumophila* that have escaped their protozoan host [177] (a). Coloured TEM of a lung macrophage containing *L. pneumophila* cells (white dots inside of purple) (b) and coloured TEM of *L. pneumophila* (c) [253].

1.4.3 Environmental ecology and route of transmission

L. pneumophila is ubiquitous in natural aquatic environments and also in manmade water systems. It appears to fail to grow alone in water either in planktonic or sessile form, however several studies have demonstrated the important role of heterotrophic biofilms in the pathogen's resistance and survival in poor environments [183, 236, 237]. The role of amoeba species is well documented and these are also ubiquitous in natural and artificial waters. As such, several groups have already demonstrated that amoeba species, including members of the genera *Acanthamoeba*, *Hartmanella*, and *Naegleria*, are not only a protective niche for the survival of *L. pneumophila* in water but also a support for their growth and multiplication [1, 187, 240, 265, 266].

There is no doubt that *L. pneumophila* is a waterborne pathogen. In fact, as no person-to-person transmission has been ever reported, water might be the only route possible, although *L. lonbeachae* has been reported to be transmitted in aerosolised dry potting compost. The infection occurs when aerosolized droplets with a diameter smaller than 8 μm are inhaled and reach the lung alveoli. This happens because droplets bigger than 8 μm are excluded by the terminal bronchiole and might be the explanation why there are not more cases of legionellosis [34]. In this way, infections are normally connected to cooling towers, whirlpool spas, fountains, shower heads, air conditioners and dental units where water contaminated by *L. pneumophila* can be aerosolized [2, 16, 34, 118, 264]. However, research by Janet Stout and colleagues has also suggested that ingestion can be a route of infection by the later microaspiration of stomach aerosols but this pathway has never been proved (cited by [143]).

The role of amoebae in the survival of *L. pneumophila* in the environment has been widely studied (Figure 1.9a). Living inside these protozoa, *L. pneumophila* will not only grow and multiply but also remain substantially protected against disinfection. The use of amoebas as a technique to resuscitate VBNC *L. pneumophila* is also commonly used in laboratories [25, 29, 37, 94, 183].

1.4.4 Diseases, diagnosis and treatment

L. pneumophila causes two kinds of disease; Legionnaires' disease, which is the pneumonia form that can be fatal, and Pontiac Fever, a milder form of the disease very similar to flu [77, 137, 169, 207]. Not all people which come into contact with *L. pneumophila* get Legionnaires' disease (or even Pontiac Fever) and symptoms are dependent on factors such as age (it is very rare in children below 15 years and much more common in people older than 40 years), gender (men are twice as susceptible than women), immunosuppression, smoking, alcoholism, autoimmune diseases and patients with chronic pulmonary disease [120, 166, 266].

The pneumonia acquired form includes symptoms such as vomiting, non-productive cough, diahrrea, myalgia, headache, rising fever and chills, bradycardia and/or confusion/delirium [120, 295]. Radiographies only show lung infection and are not specific for *Legionella*, so a proper diagnosis is achieved by the isolation and culture of samples collected from sputum lung, use of antigens in urine samples and PCR [120, 182].

The treatment of Legionnaire's disease always involves the use of antibiotics including doxycycline (if there is no certainty about the causative agent), erythtomycin, azithromycin, clarithromycin, ciprofloxacin and levofloxocin. Some studies have shown that levofloxocin is the most effective in severe pneumonias[64, 158, 175].

1.4.5 Outbreaks worldwide

Despite the fact that *Legionella* is a well known and characterized bacterium, it is estimated than on average six out of every million people get infected and outbreaks still occur worldwide (Table 1.6). The real number of *Legionella* infections is difficult to obtain, firstly because most of them will go unnoticed (Pontiac Fever is similar to a normal flu) and also because not all pneumonal diseases are checked for *Legionella*. The numbers present in Table 1.7 refer to the reported cases in the last 3 years but are certainly underestimations of the actual numbers [61, 106, 107, 114].

Table 1.6 Examples of outbreaks worldwide in the last 3 years.

Country	Month and Year	Number of cases	Number of deaths
New Zealand	June 2005	19	3
US (New York)	June 2005	21	?
Canada (Toronto)	September 2005	127	21
The Netherlands	July 2006	30	2
France	September 2006	12	0
Russia (Urals)	July 2007	150	4
UK	August 2007	5	4
US (Florida)	March 2008	2	0

Source: [106]

Legionnaires' disease is a preventable disease. The prevention and control of *Legionella* includes the adoption of guidelines (also called codes of practice, standards or best practices) which are publications resulting from the collaboration of government, academics and industrial experts. One of the best known guidelines is the "Approved code of Practice and Guidance" (often referred as "L8") developed by Health and Safety Commission (HSC, UK) now Health and Safety Executive (HSE). The publication of guidelines is vast and have been prepared by organisations such as National Health and Medical Research Council (NHMRC, Australia), Centres for Disease Control and Prevention (CDC, US), American Society of Heating, Refrigerating and Air Conditioning Engineers (ASHRAE, US), Standard Association of Australia/ Standard Association of New Zealand, European Working Group for Legionella Infections (EWGLI), World Health Organisation (WHO) to mention a few [49, 65, 79, 113].

Table 1.7 Number of cases of Legionnaires' disease in some European countries in the last 10 years.

Country	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008*
Austria	12	10	11	13	8	16	18	14	21	5
Denmark	18	13	29	18	19	33	40	26	31	6
Finland	2	4	6	7	2	4	4	6	14	1
France	28	43	85	119	120	135	158	174	181	39
Germany	1	9	19	50	48	1	0	0	0	0
Ireland	1	5	1	3	2	3	5	9	11	0
Italy	31	19	49	68	72	66	96	130	149	23
Norway	1	5	7	6	11	9	13	12	17	6
Portugal	0	0	0	0	1	7	2	5	3	0
Spain	3	10	11	33	38	30	40	73	68	28
Sweden	22	22	24	23	27	22	23	28	41	9
The Netherlands	66	103	118	152	104	119	134	158	137	29
United Kingdom[1]										
England & Wales	201 (30)	180 (24)	182 (26)	389 (33)	314 (35)	318 (38)	355 (29)	551 (52)	441 (53)	?
Northern Ireland	5(2)	1(0)	0(0)	4(0)	7(0)	5(0)	6(0)	5(0)	?	?
Scotland	35(3)	32(3)	20(2)	36(2)	29(2)	32(4)	33(1)	42(3)	?	?

Source: [80, 107, 114, 126]
[1]The numbers in brackets correspond to numbers of deaths.
*Until May

1.5 *Helicobacter pylori*

Since the identification of *H. pylori* systematic research has been done to highlight several aspects from clinical to ecological. After being discovered almost 30 years ago, its route of transmission is still unknown. It is estimated that 50% of people are infected worldwide, although most of them are asymptomatic.

1.5.1 Marshall & Warren towards the Nobel Prize

In 1982 two Australian researchers, Barry Marshall and Robin Warren, discovered that all the patients with duodenal ulcers and 80% of the patients with gastric ulcers had a common characteristic: they had a spiral-shaped non-identified bacterium present in their gastrointestinal tract. Based on these observations they suggested that the bacterium was the principal cause of gastritis and peptic ulcers [165]. During the following 10 years, research from all over the world confirmed the presence of *H. pylori* in patients with gastric ulcers. In addition, in US and Europe it was shown that the use of antibiotics against this pathogen resulted in the complete cure of the associated ulcers and their recurrence decreased by 90%. In 1994, it was established that *H. pylori* is one of the causes for the development of ulcers and this discovery resulted in a Nobel Prize in Medicine for Marshall and Warren in 2005.

The bacterium was first named *Campylobacter pyloridis* and became *pylori* after some time, due it relationship with the pylorus. A few years later, DNA tests revealed that the genomic differences between this bacterium and other *Campylobacter* species were to great for it to be considered from the genus *Camplylobacter* and the bacterium was renamed as *Helicobacter pylori*, due to its spiral shape [100].

1.5.2 Characteristics

Helicobacter belongs to the ε-proteobacteria class and consists of at least 30 species. *H. pylori* is a Gram-negative bacterium that can exist in 3 different physiological forms (spiral, U-shaped and coccoid) and grow either microaerobically (concentrations of oxygen below 10%) or anaerobically. It is a heterotrophic bacterium that uses glucose as a source of energy and carbon by its degradation to pyruvate. It is a fastidious microorganism to grow *in vitro* requiring a complex medium and several days to grow in a special gaseous atmosphere [73, 281].

This bacterium is commonly found in the epithelial cells where the pH is around 7.4. Its survival in the acid environment of the gastric lumen (pH around 2) is conferred by an enzyme that hydrolyses urea to carbon dioxide and ammonia: urease. The spiral shape and the presence of seven flagella in one of

the poles gives it high mobility, allowing it to go across the thick and viscous gastric mucosa which separates the lumen from the epithelial cells.

Although *in vivo* the spiral shape is the most predominant form, the coccoid shape has been observed and is principally associated with stress conditions. Between these 2 main shapes the bacterium passes through an intermediate U-shape (Figure 1.109). The dimensions of the spiral form range from 2-4 µm length and 0.5-0.8 µm width, but can reach 20 µm when grown *in vitro* under special conditions. The coccoid form is normally between 1 and 4 µm diameter [14]. The form in which the bacterium is might give important information about its metabolic condition. As such, *in vivo* the predominant form is spiral being therefore associated to the infective form, while the coccoid form is associated to stress conditions, such as nutrient deficiency, long incubation time, and is seen as a strategy to survival in a dormant stage. There are some authors who think that the coccoid shape is a manifestation of cellular death but it has been demonstrated that coccoid cells might be either dying, cultivable or VBNC [18, 244].

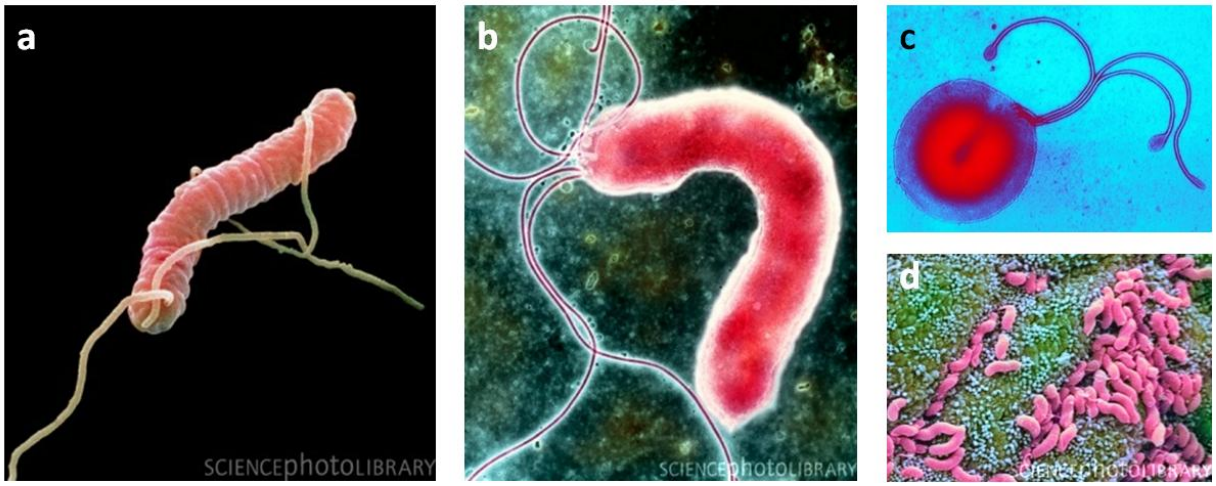


Figura 1.10 Coulored TEM of *H. pylori* in the three different physiological possible forms: spiral (a); U-shape (b) and coccoid (c). Coulored scanning electon micrograph (SEM) of *H. pylori* (pink) in the stomach lining (d) [253].

The genome of *H. pylori* comprises of approximately 1.7 Megabase pairs with 35% G+C content. One of the main genes connected to disease development is that coding for cytotoxin gene A, *cag*A [10, 73].

1.5.3 Is *H. pylori* a waterborne pathogen?

The route of transmission of *H. pylori* is still controversial but all authors seem to agree on one point; there are several routes which *H. pylori* uses to infect humans. So far, the only place from where cultivable *H. pylori* has been isolated is the gastrointestinal tract, which makes person-to-person the

favourite route. Person-to-person transmission routes include breastfeeding, gastro-oral, oral-oral, faecal-oral and iatrogenic pathways. However food ingestion and zoonotic transmission are possible as well. Table 1.8 summarizes the possible routes of transmission and respective pathways [19, 76, 99].

Table 1.8 Possible routes of transmission of *H. pylori* to humans.

Route of transmission	Way of transmission
Person-to-person	
Breastfeeding	Mothers can pass to babies through their own milk, but is more probable that increases immunity in their kids
Oral-Oral	Kissing, use of the same chopsticks, pre-masticated food
Faecal-oral	Contact with faeces
Gastro-oral	Contact with droplets of gastric juice during endoscopies, vomits and gastro-esophageal reflux
Iatrogenic	Transmission through endoscopes
Water	Ingestion of contaminated drinking water
Food	Consumption of raw vegetables, milk and derivates, meat
Zoonotic	Consumption of raw milk and meat, contact with animals

Source: [19, 76, 99]

The transmission through the ingestion of contaminated water is a possibility but despite all attempts it has not been proved yet. Several authors have detected *H. pylori* DNA by PCR either in natural reservoirs, in drinking water or associated biofilms [28, 43, 204, 222, 286] however it has been demonstrated that detection by PCR overestimates the number of cells as it does not distinguish live from dead cells [221] and therefore it is not accepted as proof that water is a route of transmission. Other authors have also detected *H. pylori* cells in drinking water biofilms by the use of a *H. pylori* specific PNA probe [39, 40] and therefore it is not accepted as proof that water is a route of transmission. Other authors have also detected *H. pylori* cells in drinking water biofilms by the use of a *H. pylori* specific PNA probe [180]. The main obstacle to prove that *H. pylori* is a waterborne pathogen is the fact that cultivable *H. pylori* has never been recovered from drinking water and molecular techniques are not accepted as they cannot prove that the *H. pylori* detected is viable. Nonetheless, it has been shown that the coccoid form of *H. pylori*, the form that is commonly found in water, is the environmental VBNC adaptation of this pathogen [18, 221] and that it is capable to recover and infect mice [47, 257]. Moreover, epidemiological studies have successfully correlated the consumption of water with the incidence of *H. pylori* [43, 123, 136, 142].

1.5.4 Diseases, diagnosis and treatment

The incidence of *H. pylori* varies from country to country and also within the same country. Even in countries with high incidence, such as Portugal, most people are actually asymptomatic and normally remain untreated until symptoms appear. *H. pylori* positive tests represent, however, a risk of development of peptic and duodenal ulcer disease, gastric B-cell mucosa associated lymphoid tissue (MALT) lymphoma and other gastric carcinoma by inducing DNA damage in epithelial cells [81, 148]. Ironically, *H. pylori* seems to have a protective effect on gastro-esophagal reflux disease, Barret esophagus and adenocarcinoma esophagus [31]. Some studies connect *H. pylori* with Crohn's disease, cardiovascular and obesity problems but they are not conclusive [162].

The diagnosis depends on clinical history, local availability and associated cost, and comprise invasive (endoscopy and biopsy) and non-invasive techniques [73]. The invasive test consists of the detection of the urease enzyme (urease test) and if positive needs to be confirmed by histological staining tests or standard culture methods. Non-invasive tests include breath tests (a solution containing urea is swallowed and the carbon dioxide in the breath is measured) and serological tests (detection of *H. pylori* antibodies in the patient's blood, serum, saliva or urine; nevertheless, this test does not distinguish past from present infection) [73, 101, 280].

Whether asymptomatic patients should undergo treatment to eradicate *H. pylori* from the stomach is subject to different opinions [86, 89]. While some physicians advise the eradication of this pathogen upon detection, some others think that treatment should only be applied when symptoms appear. In any case, the treatment consists of a triple or quadruple therapy, which includes a cocktail of two or more antibiotics (clarithromycin, metronidazole, tetracycline or amoxycillin), Triple therapy consists in the use a stomach lining shield (normally bismuth subsalicylate) or a proton pump inhibitor (cimetidine, omeprazole) and two antibiotics. In quadruple treatment both stomach lining shield and proton pump inhibitor are used in conjunction with two antibiotics [73, 95]. Several groups are at the moment trying to develop a vaccine against *H. pylori* but there are no successful results up to the present date [3, 119, 130].

1.5.5 Predominance worldwide

H. pylori occurs worldwide and affects on average approximately 50% of the world population, although the incidence has been decreasing in recent years [161, 227, 276]. Distribution appears to have a higher incidence in undeveloped countries and a low incidence in developed countries (Figure 1.11); however in Portugal and Japan, ranked, respectively, as 29th and 8th in the Human Development Index published by the United Nations Development Program [289] the incidence is higher than 80% [19, 160]. The incidence of *H. pylori* in the same country also varies accordingly if it is a developed or undeveloped country [214].

In undeveloped countries, most of the infections seem to be acquired during childhood while in developed countries the incidence increases gradually with age. In the first case, the number of children *H. pylori*-positive can reach 75% contrary to what happens in developed countries, where the prevalence is normally lower than 10% [103, 155, 161, 234].

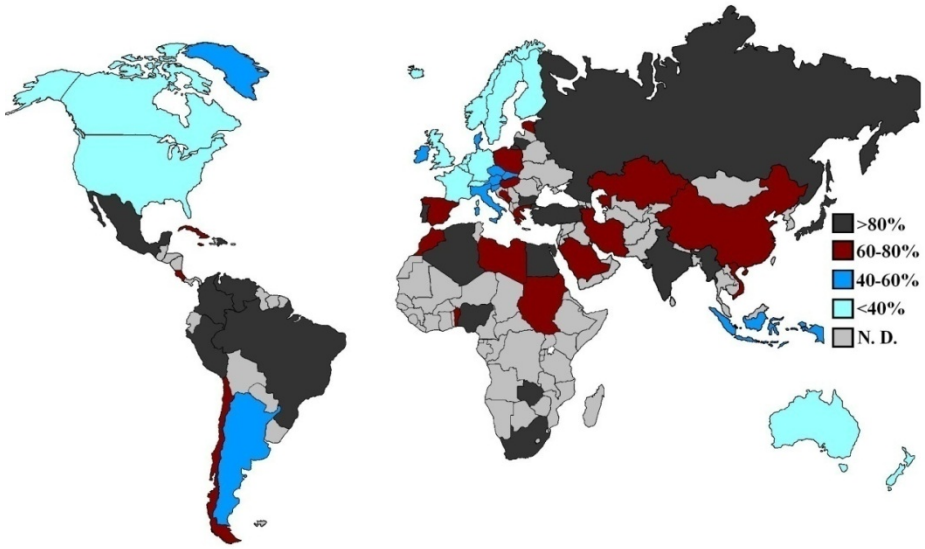


Figura 1.11 Worldwide prevalence of *H. pylori* [19].

Epidemiological studies have revealed that, in general, the high incidence of *H. pylori* is correlated with a deprivation in sanitation, hygiene and educational habits. Therefore lower socio-economical status, high population density in undeveloped countries are directly related to the high occurrence of *H. pylori* [99, 131].

1.6 Scope and purpose

As previously stated, water is a vehicle for the transmission of several pathogens and associated diseases. One of these pathogens, transmitted by contaminated water, is *L. pneumophila*. As for *H. pylori*, water might be one of the possible paths of transmission; evidence is very strong but it has not been proved yet.

The understanding of the behaviour of these two pathogens in chlorinated water and in biofilms is fundamental to the correct control of water quality. Therefore, in this work, research was undertaken to gain a better understanding of the influence of chlorine in *L. pneumophila* and *H. pylori* cells in both pure and suspended culture (as in heterotrophic biofilms) formed with microorganisms obtained from Southampton tap water. A series of experiments without chlorine were also conducted to try to highlight the role of several physico-chemical parameters in the inclusion and survival of these two pathogens inside drinking water biofilms and the influence of specific heterotrophic bacteria on the behaviour of these two pathogens in tap water and associated biofilms.

In this thesis the results are presented in the format of scientific papers, as they were submitted to peer reviewed international journals.

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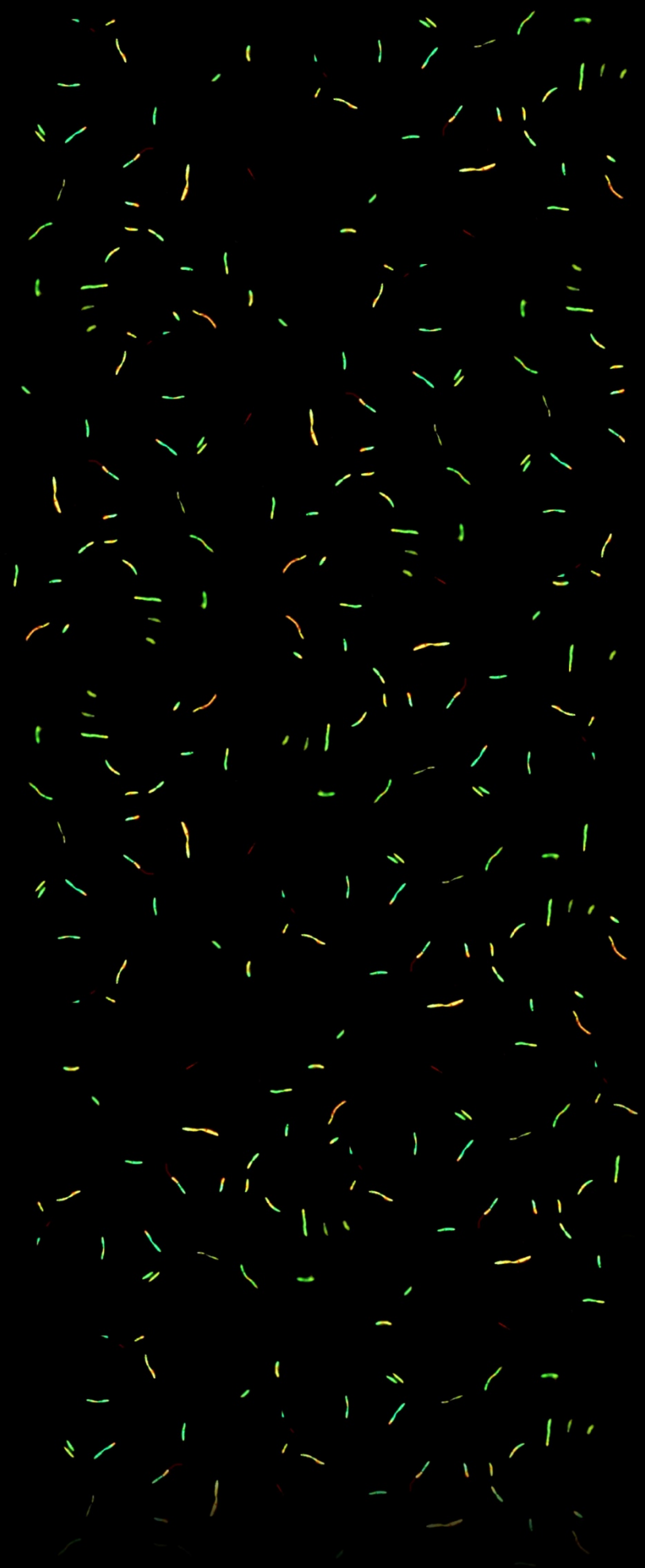

Validation of SYTO 9/ Propidium Iodide uptake for rapid detection of viable but non-cultivable *Legionella pneumophila*

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Legionella pneumophila is an ubiquitous environmental microorganism that can cause Legionnaires' disease or Pontiac fever. As a waterborne pathogen it has been found to be resistant to chlorine disinfection and survive in drinking water systems, leading to potential outbreaks of waterborne disease. In this work, the effect of different concentrations of free chlorine were studied (0.2, 0.7 and 1.2 mg l^{-1}), the cultivability of cells assessed by standard culture techniques (Buffered Charcoal Yeast Extract agar plates) and viability using the SYTO 9/Propidium Iodide fluorochrome uptake assay (LIVE/DEAD® *Bac*Light™). Results demonstrate that *L. pneumophila* loses cultivability after exposure for 30 minutes to 0.7 mg l^{-1} of free chlorine and in 10 minutes when the concentration is increased to 1.2 mg l^{-1}. However the viability of the cells was only slightly affected, even after 30 minutes exposure to the highest concentration of chlorine; good correlation was obtained between the rapid SYTO 9/Propidium Iodide fluorochrome uptake assay and a longer co-cultivation with *Acanthamoeba polyphaga* assay, confirming that these cells could still recover their cultivability. These results raise new concerns about the assessment of drinking water disinfection efficiency and indicate the necessity of further developing new validated rapid methods, such as the SYTO 9/Propidium Iodide uptake assay, to assess viable but non cultivable *L. pneumophila* cells in the environment.

2.1 Introduction

Legionella pneumophila is a Gram-negative rod shaped bacterium that can cause Legionnaires' disease (pneumonia with a high mortality rate) or Pontiac fever (a mild non-pneumonic form of illness) [19, 25, 37]. Contrary to most other pathogens that appear in drinking water due to fecal contamination [12, 32], *L. pneumophila* is known to be ubiquitous in natural aquatic systems such as groundwater, lakes and rivers [6, 9, 25]. When disinfection procedures are not effective, viable *L. pneumophila* cells can remain in water and continue to be the cause of outbreaks. This pathogen has been isolated from shower heads, whirlpools spas, cooling towers, air conditioning systems, humidifiers, etc. [9, 25, 33, 34, 35], being transmitted to humans when contaminated aerosols are formed and inhaled.

Chlorine is the disinfectant most commonly used to ensure drinking water quality and has been used since the 19th Century [28]. Comparing the disinfectants that can be used in drinking water systems, chlorine is one of the most effective as residual chlorine can remain in water to control the microbiological water quality between the application and the distribution points [14]. However, in the last few years it has been found that emerging pathogens have increased resistance to chlorine, especially *L. pneumophila*. On the other hand, studies investigating the effect of chlorine concentration on this bacterium used a simple culture method to assess viability [15, 16] which is now known to have limitations [10]. It has been demonstrated that after exposure to stress conditions, a range of different bacterial species can enter a viable but non-cultivable (VBNC) state. In this state cells are not able to grow and replicate in artificial media, but are still viable and might maintain their pathogenic properties [8, 10, 20, 23, 36, 37]. In fact it has been demonstrated that *L. pneumophila* can recover cultivability after being exposed to stress conditions when co-cultured with amoeba species [7, 30]. This procedure demonstrates that *L. pneumophila* is able to remain infective but is tedious to perform, taking several days of co-culture followed by four or more days for recovery of cultivable cells on agar media.

The aim of this work is to develop a rapid viability assay procedure and show that the assessment of *L. pneumophila* in water after exposure to chlorine stress by the use of standard methods can lead to false results: the pathogen can completely lose cultivability but still maintain membrane integrity, which we have validated to be indicative of cell viability by demonstrating infection of amoebae in co-culture.

2.2 Material and Methods

2.2.1 Strains

L. pneumophila NCTC 12821 was grown on Buffered Charcoal Yeast Extract (BCYE) agar (Oxoid, UK) for 24 hours at 30ºC. Cells were suspended in 50 ml of dechlorinated and filtered tap water to give a final concentration of approximately 10^7 cells ml^{-1}.

2.2.2 Chlorine preparation and measurements

Chlorine tablets (H-8801, Guest Medical, UK) were added to filtered distilled water to obtain a 5 g l^{-1} stock solution. The measurement of chlorine was done using the N,N-dimethyl-p-phenilenediamine (DPD) colorimetric method, as described in the Standard methods for the examination of water and wastewater [2] with the exception of the absorbance wavelength reading which was adjusted to 492 nm [21].

2.2.3 Chlorine disinfection tests

After considering the chlorine demand due to organic matter, an appropriate amount of the stock solution was added to the suspension in order to obtain a final concentration of free chlorine of 0.2, 0.7 and 1.2 mg l^{-1}. A control assay with no chlorine addition was also performed. Experiments were carried out in amber flasks (to avoid chlorine degradation by light) at room temperature (20ºC) and stirred at 620 rpm. Samples were taken at 0, 10, 20 and 30 minutes and cells quantified as explained below. At times 0 and 30 minutes the concentration of free chlorine was measured by the DPD method as described previously. The chlorine reaction was inactivated by the addition of sodium thiosulfate (Sigma, UK) applied at a final concentration of 5 mg l^{-1}. For each chlorine concentration the experiment was repeated at least three times.

2.2.4 Assessment of cultivable cells

A 40 μl aliquot of each sample was diluted (to give between 15 and 150 colony forming units (CFU) per agar plate) and spread onto BCYE agar plates (in triplicate for each experiment) and aerobically incubated at 30ºC for 4 days. After this time the number of colonies was counted to determine the number of cultivable cells remaining in the chlorinated solution. When, after 4 days, no colonies were grown on BCYE agar plates, the plates were returned to the incubator for 14 days. Using this method the limit of detection is 8.33 CFU ml^{-1}.

2.2.5 Assessment of membrane integrity

To assess the membrane integrity the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit (Molecular Probes, Invitrogen, UK) was used. A 50 μl aliquot of each sample was diluted in 0.950 ml of dechlorinated, filtered tap water and stained with SYTO 9/Propidium Iodide (PI). A 3 μl volume of an equal proportion of SYTO 9 and PI mixture was added to the sample and incubated in the dark, at room temperature (20ºC) for 15 minutes followed by filtration through a black polycarbonate Nucleopore® membrane (0.2 μm pore size) (Whatman, UK). Subsequently, the membranes were air dried, mounted onto glass slides with non-fluorescence immersion oil and a cover slip. The slides were examined using a Nikon Eclipse E800 episcopic differential interference contrast/epifluorescence (EDIC/EF) microscope under oil immersion (Best Scientific, UK) [13].

2.2.6 Co-culture of *L. pneumophila* and *Acanthamoeba polyphaga*

An axenic culture of *A. polyphaga CCAP1501/18* was maintained in Proteose Peptone Glucose Medium (PPG; CCAP, UK) at room temperature and subcultured every week. Five ml samples of the suspension exposed to 1.2 mg l^{-1} of free chlorine for 0 (control) and 30 minutes were centrifuged at 3000 rpm for 10 minutes (Heraeus, UK) and washed three times in PP medium (PPG but with glucose omitted) and before resuspending in a final volume to achieve the concentration of approx. 5 x 10^5 cells ml^{-1}. Infection of *A. polyphaga* by *L. pneumophila* was performed as described by Garcia et al. [7]. Briefly, monolayers of *A. polyphaga* were formed in 96 well-plates in the presence of PP medium at a concentration of 10^4 cells per well and infected with 200 μl of the *L. pneumophila* suspension prepared as described above. The plates were then centrifuged at 500 x g for 5 minutes and incubated at 30ºC for 1 hour. After this time plates were washed three times with PP medium and incubated at 30ºC for 1 hour in 50 μg ml^{-1} of gentamicin followed by three washes with PP media. The infected monolayers were then incubated at 30ºC and the cultivability of *L. pneumophila* was assessed after 24, 48 and 72 hours of infection. For that, *A. polyphaga* cells were lysed with 0.05% (v/v) Triton X-100 (Sigma, UK) and supernatants before and after lysis were combined and 40 μl aliquots were plated onto BCYE as described above.

2.2.7 Assessment of RNA injury

A 1.0 μl aliquot of SYBR® Green II RNA gel stain (SYBR II) (Molecular Probes, Invitrogen, UK) was added to 50 μl of each sample diluted in 0.95 ml of dechlorinated filtered tap water and incubated in the dark, at room temperature (20ºC) for 30 minutes. The stained suspension was then filtered through a black polycarbonate Nucleopore membrane (0.2 μm pore size), air dried and mounted onto glass slides with non-fluorescence oil and cover slips and examined using EDIC/EF microscopy [13].

2.2.8 DNA electrophoresis

L. pneumophila NCTC 12821, grown under the same conditions as previously described, was suspended in 100 ml of dechlorinated and filtered tap water to give a final concentration of approximately 10^7 CFU ml^{-1}. This cell suspension was exposed to the same range of chlorine concentrations for 30 minutes. Following this, cells were concentrated by centrifugation at 4000 rpm for 10 minutes and the DNA extracted and purified using a DNA extract kit (Sigma, Spain). The DNA obtained was run in a horizontal electrophoresis system for 2 hours at 100 V using 1% (w/v) agarose gel (Bio-Rad, Portugal) containing ethidium bromide (50 $\mu l\ l^{-1}$ of a 10 mg ml^{-1} stock) (Bio-Rad, Portugal). Finally, the gel was visualized under UV light.

2.2.9 Statistical analysis

Results obtained for cultivable cells, membrane integrity, RNA injury and total cell counts were transformed on a logarithmic scale. The average for each was calculated from at least three experiments, and the homogeneity of variances across these parameters was checked by the Levene test for equality of variances using a statistical package (SPSS Inc., Chicago IL, USA). Differences between the parameters measured were subsequently compared by a one-way ANOVA followed by a Bonferroni post hoc test. Differences were considered relevant if $P<0.05$.

2.3 Results

The chlorine concentrations measured in the cell suspension at time 0 and 30 minutes indicated that there was consumption of chlorine by reaction with *L. pneumophila* cells, as indicated in Table 2.1. Preliminary results showed that there was consumption of chlorine by the organic matter present in the tap water (results not shown), so the values presented (0.2, 0.7 and 1.2 mg l^{-1}) are the values following subtraction of chlorine consumption by organic matter. However, at time 0 minutes the values obtained were much lower than theoretically expected (i.e. compared to values obtained in tap water without cells), meaning that there was immediate consumption by cells.

Table 2.1 Chlorine concentration demand immediately after, and 30 minutes after, the chlorine addition to the sterile-filtered tap water and to the *L. pneumophila* suspension.

Cl_2 concentration (mg l^{-1})	**Cl_2 measurement in the cell suspension (mg l^{-1})**	
	0 min	**30 min**
0.2	0.058	0.030
0.7	0.483	0.083
1.2	0.858	0.140

The effect of chlorine on *L. pneumophila* cells was evaluated by quantification of cells by two different methods: standard culture techniques and direct count of cells by observation under epifluorescence microscopy after staining with the SYTO 9/PI fluorochrome reagents (Figure 2.1).

The assay where no chlorine was added to the suspension served as a control and showed that cells maintain their physiological state in dechlorinated filtered tap water for at least 30 minutes. In fact, ANOVA results show that time does not influence any of the parameters studied, including cultivability ($P>0.95$). Cultivable cells represented 55% of the total number of cells and, as expected, differences between cultivability and either the total number of cells or viable cells (assessed by SYTO 9/PI membrane integrity staining) were statistically significant ($P<0.05$). When cells were exposed to 0.2 mg l^{-1} chlorine there were no alterations in the viability status but the cells appeared to start losing their cultivability in the first 10 minutes. This result was confirmed by the statistical analysis on the effect of time on this parameter ($P<0.05$). Increasing the chlorine concentration up to 0.7 mg l^{-1} caused a complete loss of growth capacity on agar plates (total loss of cultivability). Although no statistically significant differences were detected between the number of viable and total cells ($P>0.05$), it was observed that some of the cells did not fluoresce true green but had become yellow/orange; however only cells fluorescing red were considered as dead cells due to a compromised cell membrane. Cells exposed to the maximum concentration of chlorine (1.2 mg l^{-1}) showed a loss of cultivability during the

first 10 minutes of chlorination and it was observed that there was an increase in the number of red fluorescent cells. Therefore, at this concentration, the difference in numbers between viable and total cells was statistically significant (P<0.05).

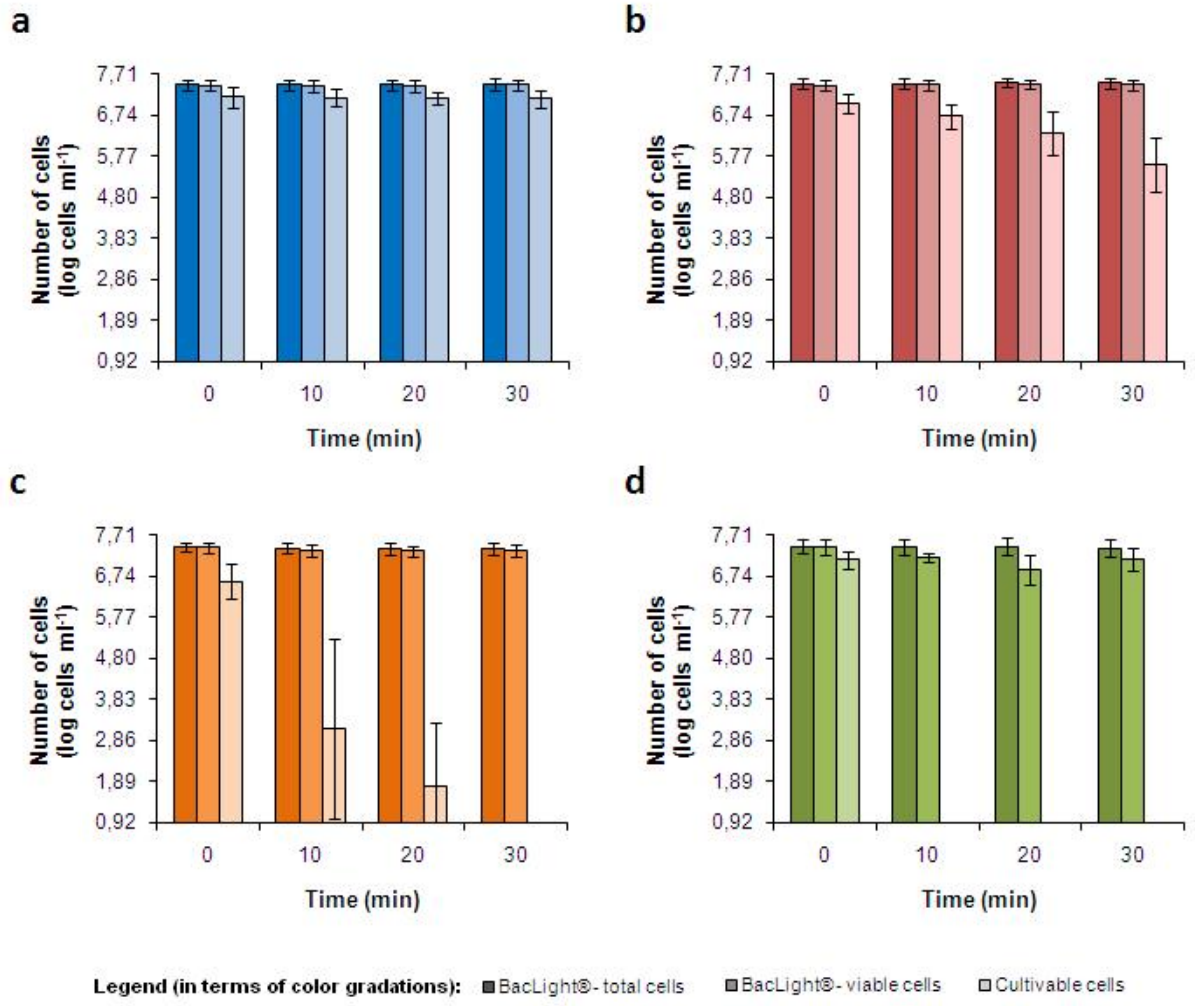

Figura 2.1 Variation in the total cell number, viability of SYTO 9^{-}/PI^{+} stained cells and cultivability on BCYE agar, after exposure to free chlorine concentrations of 0.0 (a), 0.2 (b), 0.7 (c) and 1.2 (d) mg l^{-1}. Error bars represent standard deviation of at least three experiments.

To validate the results obtained with the LIVE/DEAD viability kit a sample of *L. pneumophila* exposed to 1.2 mg l^{-1} of free chlorine for 30 minutes was treated and co-cultured with *A. polyphaga*. Figure 2.2 shows that cells were able to recover cultivability between 24 and 48 hours of infection. Simultaneously, a control experiment was performed in which cells not stressed with chlorine were also co-cultured with *A. polyphaga*. After 72 hours of co-culture the numbers of cultivable cells were lower than before exposure to *A. polyphaga*.

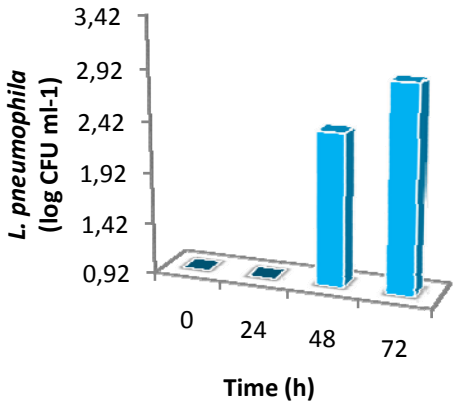

Figura 2.2 Number of cultivable *L. pneumophila* after 24, 48 and 72 hours of co-culture with *A. polyphaga*. 0 hours represents the number of cultivable *L. pneumophila* after 30 minutes of exposure to 1.2 mg l^{-1} of free chlorine.

To investigate if *L. pneumophila* cells lost cultivability so quickly due to nucleic acid damage, chlorine treated cells were stained with the SYBR II fluorochrome. This stains nucleic acids although fluorescence intensity is much stronger when it binds to RNA compared to double stranded DNA [17]. It was observed that neither fluorescence intensity nor cell numbers changed with increasing chlorine concentrations suggesting that there are no variations in the RNA content in the cells under these conditions (results not shown). The DNA bands obtained by electrophoresis also show that there are no changes in the DNA content (results not shown).

2.4 Discussion

All the experiments described here were carried out by suspending the cells in dechlorinated, filtered tap water to represent realistic conditions. As expected when added to tap water (with no previous inoculation), there was a decrease in the chlorine concentration. This happens because tap water contains organic matter that can react with chlorine, the so-called chlorine demand, meaning that the real concentration available to interact with *L. pneumophila* cells is lower than the concentration added to the suspension. Prior studies showed that the chlorine consumed by organic matter is approximately 0.3 mg l^{-1} and occurs rapidly in the first 10 minutes (results not shown).

When chlorine was added to the *L. pneumophila* suspension, a sample was immediately taken and analyzed (corresponding to time zero). As seen in Table 2.1, a high proportion of the chlorine reacts instantly, with values significantly lower than the concentration added. The results obtained in tap water with no cells show a lower reduction confirming that chlorine reacts with the cells and that the reaction starts immediately after the addition. This can also explain the rapid loss of cultivability in the first 10 minutes after the addition of 1.2 mg l^{-1} of free chlorine, as seen in Figure 2.1.

The *L. pneumophila* cell suspensions were prepared using a 24 hour culture so each batch of cells were in the same physiological conditions. To control this parameter, and because the chlorine reaction with cells seemed to be immediate and fast, a sample was taken before chlorine addition and cells were quantified by spreading on BCYE agar plates and by SYTO 9/PI double staining. SYTO 9/PI can be successfully used to stain *L. pneumophila* cells, as previously demonstrated by Ohno et al. [22]. Although the cultivability was found in the present study to be slightly variable, these values do not seem to be significant, the viability results showed that the cells were in a very similar state (percentage values of viable cells were always around 95 % of total cells).

In the absence of chlorine there was no loss of cultivability and viability of the cells in tap water. This was expected as *L. pneumophila* can survive for long periods in tap water [11, 29, 36] and can even grow and replicate under particular conditions [35, 38].

When 0.2 mg l^{-1} of free chlorine were added to the suspension it was found that *L. pneumophila* cells lost some cultivability but there were cells that could still be recovered by standard culture techniques. Microscopy observation of cells stained with SYTO 9/PI revealed that the number of viable cells was constant with time and also that the cells maintained a bright green color. The chlorine measurement after 30 minutes demonstrated that all chlorine was consumed by the cells. In fact it can be considered that, at this concentration, chlorine is completely consumed immediately after its addition, as can be seen on Table 2.1 where the value at 0 minutes is very close to 0 and after 30 minutes no chlorine remains in the solution. The fact that some cells can still be grown on agar plates demonstrates that this level of chlorine represents a sublethal concentration and as such does not cause any damage to the membrane integrity since no cells take up PI. The loss of cultivability when cells are exposed to stress conditions (such as extreme temperatures, nutrient starvation or chlorine oxidative stress

exposure) is an effect which can be explained by injury to the cytoplasmic membrane and transport processes which reduce the membrane potential and respiratory activity, and consequently cultivability [1, 3, 18]. However, Yamamoto et al. [37] have already demonstrated that even after losing cultivability due to nutrient starvation cells retain intact DNA and RNA and in favorable conditions can recover, elongate and multiply.

When the free chlorine concentration was increased up to 0.7 mg l^{-1} the cells lost cultivability completely between 20 and 30 minutes of exposure time; however, the number of viable cells assessed using SYTO 9/PI was not altered. Although the green cells lost their brightness and some of them became yellow and orange, there was no increase in the number of cells that fluoresced red. Some authors have suggested that when cells change their fluorescence color from green to orange or yellow but are not exactly red, this means that there is some injury to the cellular membrane that allows some of the PI to penetrate the cell. However when the injury is minor the concentration of PI that can penetrate the cell and bind to DNA is not high enough to exclude all the SYTO 9 in the cell bound to DNA, so they appear yellow and orange, and are considered as viable cells [5]. Indeed, no residual free chlorine remained at the end of the incubation period which might account for the extended viability of the cells.

The maximum chlorine concentration used (corresponding to 1.2 mg of free chlorine l^{-1}) was sufficient to cause complete loss of cell cultivability in 10 minutes but once again there was an insignificant decrease in viability. In contrast it was observed that most of the cells were not green or red, but orange which suggests that low level injury to the cytoplasmic membrane had occurred due to the chlorine concentration. At the end of these experiments some free residual chlorine was still detectable, indicating that this concentration of chlorine was in excess of that needed to completely react with the cells within 30 minutes.

The use of LIVE/DEAD to assess the viability of cells is still controversial. Some authors are sceptical in accepting that green cells that have lost their cultivability are effectively viable [4, 31]. To validate the results obtained in this work, a sample of *L. pneumophila* previously exposed to 1.2 mg l^{-1} of free chlorine for 30 minutes was used to infect *A. polyphaga*. Results demonstrated that *L. pneumophila* has, in fact, entered into a viable but non-cultivable state as cells recovered their capability of growth on artificial media (BCYE) between 24 and 48 hours of co-culture with amoebae. Alternatively, in the control, where cells were not exposed to chlorine, the number of cultivable cells, after 72 hours, was lower than before co-culture with amoebae, which indicates that cells were not multiplying but were instead resuscitating inside of the amoebae. These results clearly demonstrate that *L. pneumophila* cells that appear green when stained with *Bac*Light™ kit were effectively viable, although not cultivable. The resuscitation to a cultivable state of VBNC *L. pneumophila* using amoeba species has been demonstrated before [7, 30] and therefore used in this work to demonstrate that LIVE/DEAD is a technique that can be successfully used to access the effectiveness of disinfection. The advantage of this assay is the short time it takes to obtain the results: using this method the results can be obtained in a few hours while using the amoeba co-cultivation assay the results are not available in less than one week.

The resuscitation of VBNC cells that have been exposed to stress conditions such as chlorination is sometimes difficult to achieve. Oliver et al. failed to resuscitate chlorine treated *E. coli* cells when using several methods previously demonstrated as successful [24]. However, and unlike the present work, they have not used live eukaryotic hosts for resuscitation. It has been clearly demonstrated here that *L. pneumophila* very quickly loses cultivability without losing viability, which is not surprising as it had been demonstrated previously that non-cultivable cells can still be viable [8, 22]. Moreover, Hussong et al. [10] demonstrated that non-cultivable *L. pneumophila* cells are not just viable but can cause infection of embryonated eggs. On the other hand, Steinert and colleagues [30] had suggested that Pontiac fever, a mild from of disease caused by *L. pneumophila*, could be due to VBNC cells.

At all concentrations of chlorine investigated (0.2, 0.7 and 1.2 mg l^{-1}) the number of total cells remained constant during the 30 minutes of experimental time. SYTO 9 and PI are both fluorochrome stains that bind with nucleic acids and when there is some damage to the nucleic acids they are not able to bind and the cells can not be visualized. The fact that the number of total cells observed by epifluorescence microscopy was always the same suggests that there was little or no injury to the nucleic acid structure, as expected at this low concentration of free chlorine. These results were corroborated by DNA electrophoresis which indicated that genomic DNA remained intact following chlorine treatment (results not shown). Although Phe et al., [26, 27] have previously shown that chlorine is able to damage the nucleic acids in *Escherichia coli* cells the disinfectant dosage used was much higher and, in addition, it is already known that *L. pneumophila* is more resistant to chlorine than *E. coli*. [16].

This study has clearly demonstrated that the standard culture methods used to assess the presence of *L. pneumophila* are not ideal to study the presence of this pathogen and especially its viability, because even after completely losing the capability of growth on BCYE agar plates, cells remained viable and able to infect amoebae. This raises a new concern for water quality assessment and requires the development, as reported here, of new validated, rapid methods to detect viable *L. pneumophila* cells in drinking water after disinfection.

In addition, it also suggests that viable *L. pneumophila* are probably more widespread in drinking water distribution systems than previously thought, despite the presence of residual chlorine at concentrations indicated by the international health protection agencies to control microbiological quality. The fact that a relatively low number of outbreaks with this bacterium are observed is perhaps more related to the fact that for disease to occur in humans, unlike many waterborne pathogens causing gastrointestinal infection, *L. pneumophila* has mainly to be inhaled in the form of aerosols to gain access to the lung.

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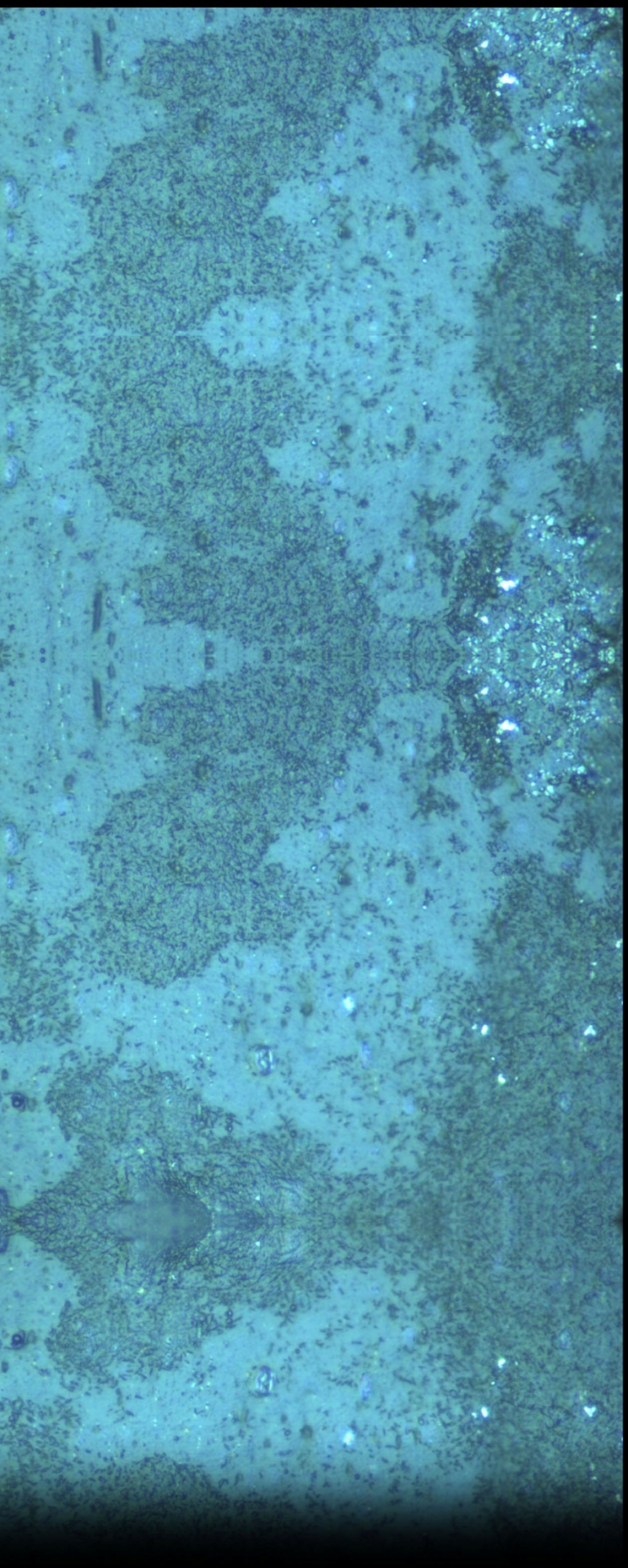

Comparison between standard culture and fluorescence *in situ* hybridization methods to study the influence of physico-chemical parameters on *Legionella pneumophila* survival in drinking water biofilms

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Legionella pneumophila is a waterborne pathogen that is mainly transmitted by the inhalation of contaminated aerosols. In this work, the influence of several physico-chemical parameters relating to the supply of potable water were studied, using a *L. pneumophila* PNA specific probe, to quantify total *L. pneumophila*, in addition to standard culture methods. To form the heterotrophic biofilms a two-stage chemostat was used, with biofilm generating vessels fed with naturally occurring *L. pneumophila*. The substratum was the commonly used potable water pipe material, uPVC. It proved difficult to recover cultivable *L. pneumophila* due to the overgrowth of other microorganisms and/or the loss of cultivability of this pathogen. Nevertheless, results obtained for total *L. pneumophila* numbers using the specific PNA probe showed that for the two temperatures studied (15 and 20ºC) there were no significant differences when shear stress was increased. However, when a source of carbon was added there was a significant increase in numbers at 20ºC. Comparing the two temperatures it was observed that at 15ºC the numbers for total *L. pneumophila* are in general higher compared to the total microbial flora, suggesting that lower temperatures support the inclusion of *L. pneumophila* in drinking water biofilms. This work also demonstrates that standard culture methods are not accurate in the evaluation of water quality when considering *L. pneumophila* and raises public health concerns since culture methods are still considered to be the gold-standard to assess the presence of this opportunistic pathogen.

3.1 Introduction

Legionella pneumophila is an ubiquitous pathogen that, after infecting humans, will cause one of two forms of disease; Legionnaire's disease, the pneumonia form that can be fatal, and Pontiac's fever, a mild form of the disease similar to influenza [18, 20]. The route of transmission is mainly by inhalation of contaminated aerosols [4, 24] and, being a waterborne pathogen, contamination of drinking water plays a very important role in the outbreaks that still occur worldwide. In fact, this pathogen has been isolated from drinking water distribution systems (DWDS), especially associated with biofilms [7, 11] and is considered to be chlorine resistant [14, 15].

It is well known that several environmental conditions, such as temperature, iron concentration, carbon source availability, plumbing materials, etc., can significantly influence the presence of *L. pneumophila* in water and biofilms. However, all studies reported use standard agar plating methods (cultivability) as a way to assess *Legionella* numbers [21, 22, 23, 25]. Under stressful conditions various pathogens, including *L. pneumophila*, can enter a viable but non-cultivable (VBNC) state where the bacteria are not able to grow directly on nutritious and selective medium [8, 10, 19, 26]. Moreover, when *L. pneumophila* is sampled from its natural environment it is difficult to recover on artificial plating media and is hence considered a fastidious microorganism [4]. In the last few years, molecular techniques have been developed to try to overcome this non-cultivability problem, including the use of peptide nucleic acid (PNA) specific probes which can identify specific microorganisms in complex microbial consortia including biofilms [1, 17, 27]. Recently, it has been demonstrated that the PNA probe PLPNE620 can be successfully used to specifically detect *L. pneumophila* in drinking water biofilms [27].

The aim of this work is to study and compare the influence of different physico-chemical parameters, such as temperature, carbon concentration and shear stress, on the survival of total and cultivable *L. pneumophila* in heterotrophic biofilms.

3.2 Material and Methods

3.2.1 Biofilm experiments

The formation of biofilms was carried out using a two-stage chemostat model system (Figure 3.1a) [12]. The first stage consisted of a 1-litre vessel (seed vessel) and the second stage consisted of three 1-litre vessels running in parallel, but connected in series with the seed vessel. All chemostats were autoclaved and filled with filter-sterilized (0.2 μm pore size Nylon filter) tap water (1-litre). The seed vessel was then inoculated with a microbial consortium that was obtained from tap water by filtration through a 0.2 μm pore size Nylon filter (Pall Gelman, UK). Preliminary experiments have shown that there was autochthonous *L. pneumophila* in the chemostats, by using the specific *L. pneumophila* PNA probe. The seed vessel was maintained in batch mode for two days to promote microbial growth and then changed into a continuous mode, being fed with filter-sterilized and dechlorinated tap water at a flow rate of 50 ml h^{-1}. This chemostat was operated at room temperature and stirred at 300 rpm to ensure that the oxygen and nutrient concentrations were homogeneous. The effluent was divided in three and used to feed the second stage chemostats; the biofilm-growing vessels. Each biofilm-growing vessel was also fed with fresh medium (filter-sterilized tap water) at a flow rate that maintained a dilution rate of 0.2 h^{-1} to promote typical environmental conditions for biofilm growth. The first vessel, where no carbon source was added, was stirred at 300 rpm and served as a control. The second vessel was stirred at 1200 rpm but with no carbon addition (high shear stress) and the third vessel was stirred at 300 rpm and 8.8 mg l^{-1} of carbon was added by the inclusion of 30 mg l^{-1} of sodium acetate to the fresh medium (high carbon concentration). The temperature was controlled at either 15ºC or 20ºC by a proportional integral derivative unit system (Brighton Systems, UK). The first temperature has been suggested to be a key temperature at which the cultivability of several waterborne microorganisms starts to be affected [3], whereas the second is generally considered to be the temperature of the drinking water during summer months. After 10 days, conditions in the biofilm-growing vessels were stable and sterile unplasticized polyvinylchloride (uPVC) coupons could be immersed (day 0). The coupons were removed after 1, 2, 4, 8, 16 and 32 days, gently rinsed to remove planktonic cells attached to the surface of the biofilm, and scraped to quantify sessile cells Figure 3.1a).

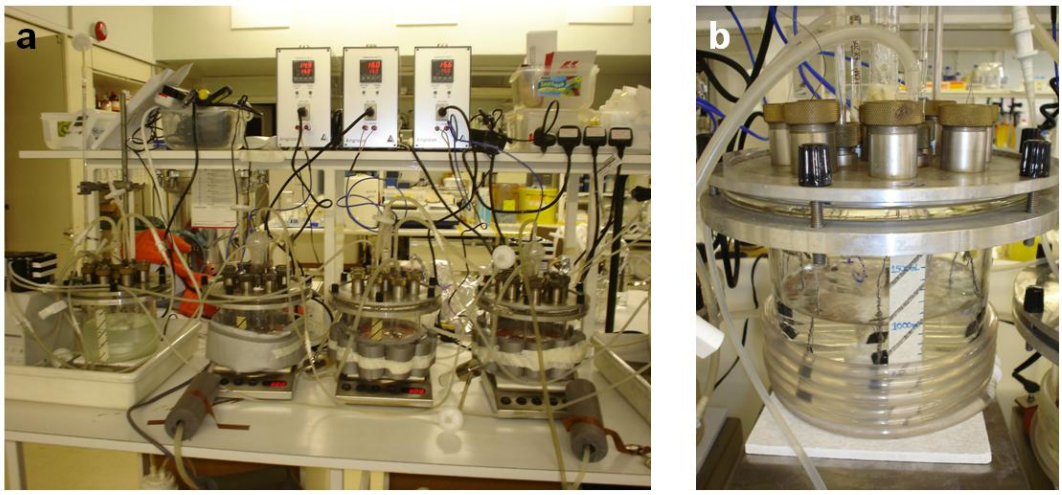

Figura 3.1 Photograph of the two stage chemostats installation (a) and a vessel with the coupons (b).

3.2.2 Treatment of coupons

One cm^2 uPVC coupons were used as a support for biofilm growth. Coupons were immersed in water and detergent for 5 minutes, washed with a bottle brusher, rinsed twice in distilled water and air-dried. Subsequently, they were washed in 70% (v/v) ethanol to remove any organic compounds, attached to the end of a titanium wire and autoclaved [12].

3.2.3 Quantification of planktonic cells

Water samples were taken after 0, 1, 2, 4, 8, 16 and 32 days from the seed and biofilm-growing vessels and were analyzed for total cells, heterotrophic cells and cultivable *L. pneumophila*. Total cells were quantified using SYTO 9 (Molecular Probes, Invitrogen, UK). To do this, 1 ml of an appropriate dilution was mixed with 0.5 μl of SYTO 9, incubated in the dark for 15 minutes, filtered through a 0.2 μm pore size polycarbonate black Nucleopore® membrane (Whatman, UK) and allowed to air-dry. Then a drop of non-fluorescence immersion oil (Fluka, UK) and a coverslip were added before observation under a Nikon Eclipse E800 episcopic differential interference contrast/epifluorescence (EDIC/EF) microscope (Best Scientific, UK) [13]. As the cells were homogenously distributed, fields of view were chosen at random and the number of cells counted on each membrane. HPC were quantified by plating onto R2A medium (Oxoid, UK) and incubated at 22ºC for 7 days. Cultivable *L. pneumophila* was quantified by plating onto Buffered Charcoal Yeast Extract (BCYE) agar plates (Oxoid, UK) and *Legionella* selective medium consisting in BCYE supplemented with glycine, vancomycin, polymixin and cycloheximide (GVPC) (Oxoid, UK) and incubated at 30ºC for up to 14 days.

3.2.4 Quantification of sessile cells

Coupons were immersed in 10 ml of filter-sterilized tap water containing autoclaved 2 mm diameter glass beads (Merck, UK) and vortexed for one minute to remove all the biofilm from the coupons surface and homogenize the suspension. Total cells, HPC and cultivable *L. pneumophila* were quantified using the methods described above. In addition, total *L. pneumophila* were quantified using the specific PNA probe PLPNE620 (5'-CTG ACC GTC CCA GGT-3') (Eurogentec, Belgium) in a fluorescence *in situ* hybridization assay (PNA-FISH) [27]. PNA-FISH was carried out by taking 1 ml of an appropriate dilution and filtered through a 0.2 μm anodisc membrane (Whatman, UK). This was left to air dry. Then the membrane was covered with 90% (v/v) ethanol to fix the cells and again air dried. The hybridization, washing and microscopy observation method was performed as described by Wilks and Keevil [27].

3.2.5 Confirmative tests

To verify which of the microorganisms isolated on BCYE were effectively *L. pneumophila* a loop of each strain was resuspended in filter-sterilised tap water and plated onto BCYE, BCYE with no L-cysteine addition (BCYE –cys) and GVPC before after acid and heat treatment. Acid treatment consisted in mixing 500 μl of HCl – KCl buffer with 500 μl of sample followed by incubation at room temperature for 5 minutes as described in the International Standard ISO 11731 [9]. Heat treatment was performed by placing a 1 ml Eppendorf tube containing each sample in a water bath at 50ºC for 30 minutes [9]. All bacteria isolated on R2A or BCYE media were identified by 16S DNA sequencing at DNAVision SA (Belgium). Briefly, for each culture DNA was purified, amplified and sequenced with 16S primers. The analysed fragments were about 1600 base pairs length. The sequenced fragments were then BLASTed in the NCBI public database and therefore obtained the Genbank accession number for the highest sequence similarity value.

3.2.6 Statistical analysis

The homogeneity of variances of total number of cells, total *L. pneumophila*, HPC and relation between *L. pneumophila* of cells and total cells was checked by the Levene test for equality of variances using a statistical package (SPSS Inc., Chicago IL, USA). Differences were subsequently compared by a one-way ANOVA followed by a Bonferroni post hoc test. Differences were considered relevant if $P<0.05$.

3.3 Results

3.3.1 Microbial dynamics in the seed vessel

During the entire experiment, the seed vessel was operated continuously at room temperature and stirred at 300 rpm; the microbial community stabilized within several days of operation and the numbers of total cells and HPC averaged 5.01 x 10^6 cells ml^{-1} and 1.15 x 10^6 colony forming units (CFU) ml^{-1}, respectively. No cultivable *L. pneumophila* was recovered on BCYE; however a previous assessment using PNA-FISH has revealed the presence of autochthonous *L. pneumophila* in biofilms, indicating the presence of this pathogen in the seed vessel.

3.3.2 Planktonic cells in the biofilm-growing chemostats

In a first series of experiments the temperature of the biofilm-growing vessels was maintained at 20ºC and the influence of shear stress and carbon concentration was studied. In Table 3.1 the average values for total cells and HPC is presented. At this temperature, shear stress does not significantly influence the number of total planktonic cells ($P>0.05$); however when a carbon supplement is added this number increases by almost 1-log, a statistically significant increase ($P<0.05$), indicating that the culture is carbon limited. In terms of cultivability some differences can be detected for the three conditions tested. The HPC numbers obtained for high shear stress are slightly higher compared to the control ($P>0.05$) but significantly lower compared to the high carbon ($P<0.05$).

Table 3.1 Average numbers of total cells and HPC in the planktonic phase at 20ºC and 15ºC for all three conditions tested.

	Total cells x 10^{-6} (cells ml^{-1})	**HPC x 10^{-6} (CFU ml^{-1})**
T = 20ºC		
LS/LC	3.21	1.13
HS/LC	2.90	2.00
LS/HC	16.4	5.51
T = 15ºC		
LS/LC	3.39	1.19
HS/LC	2.44	0.63
LS/HC	15.3	2.27

When the temperature was changed to 15ºC, the differences between the different conditions were significant for both total cells and HPC numbers ($P<0.05$). Table 3.1 shows that the number of total planktonic cells were, on average, almost 1-log higher in the high carbon chemostat comparing to the control and high shear stress.

At both temperatures, the recovery of planktonic cultivable *L. pneumophila* was never possible for any of the conditions tested, due to the overgrowth of other microorganisms and/or the loss of cultivability by this pathogen.

Comparing the results obtained under the same conditions but for the two different temperatures, it was verified that temperature had no influence on the total number of cells ($P>0.05$). Conversely, there was an important decrease in the numbers of HPC in the vessels operated at a high shear stress and high carbon concentration when the temperature was decreased ($P<0.05$).

3.3.3 *L. pneumophila* in heterotrophic biofilms

Figure 3.2a shows the results obtained for biofilm experiments at 20ºC. In terms of total cells it is possible to observe that the curves for the three conditions tested overlap, meaning that the differences between the three conditions tested gave similar results ($P>0.05$). The number of total *L. pneumophila* quantified using the specific PNA probe, were not statistically different between the control and high shear ($P>0.05$) but significantly different comparing these parameters to the high carbon environment ($P<0.05$), indicating that high concentrations of carbon are the most favorable condition to support sessile numbers of *L. pneumophila*, followed by the increase of shear stress. The increase of carbon concentration also had a great effect on sessile HPC numbers ($P<0.05$) but the increase of shear stress seems to have little effect on HPC numbers ($P>0.05$).

Figure 3.2b shows the results obtained for the biofilm experiments at 15ºC. At this temperature, it is possible to observe that the numbers of total cells vary slightly during biofilm formation and between the different conditions tested (what is also indicated for the value of $P>0.05$). Concerning the total *L. pneumophila*, the numbers tended to be slightly higher when carbon was added ($P>0.05$). Apparently the differences between the three conditions tested are only noticeable in terms of cultivable cells as it was in the numbers of HPC that it was possible to obtain great differences ($P<0.05$).

Comparing the results obtained at 20ºC and 15ºC it was possible to observe that total cells and total *L. pneumophila* numbers are quite different ($P<0.05$) while the HPC numbers are similar ($P>0.05$). In general the total cells and the total *L. pneumophila* present in the biofilm were almost 1-log higher at 15ºC in all the conditions tested. When cultivable cells are compared, there were only significant differences for HPC in the control experiment ($P<0.05$).

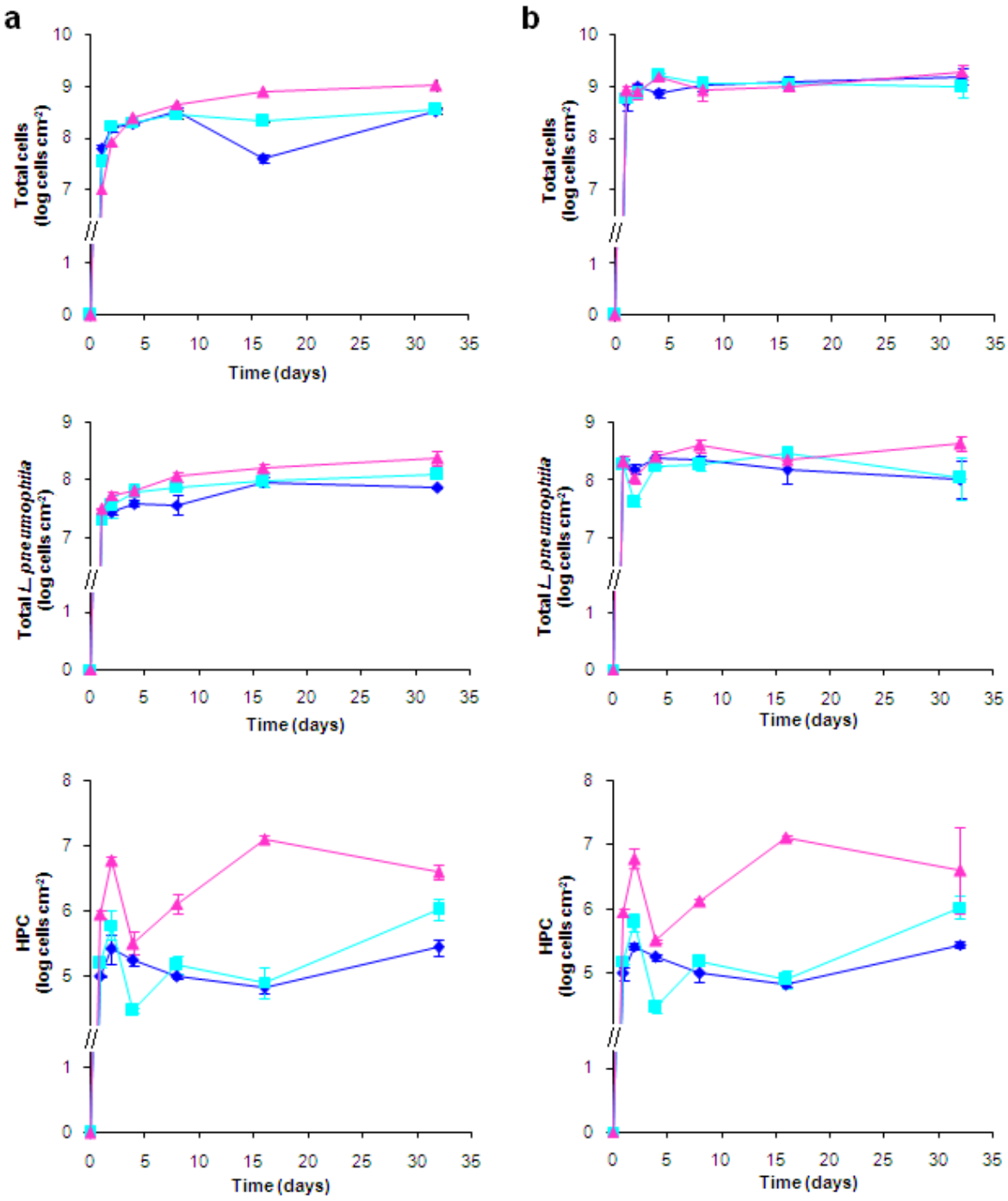

Figura 3.2 Variation in the total cell number, total numbers of *L. pneumophila* and HPC in biofilms formed at 20ºC (a) and 15ºC (b) in the control (◆), high shear stress (■) and high concentration of carbon (▲).

In previous studies, the application of PNA probes to detect specific pathogens *in situ* (i.e. conducted directly on the coupon substratum) was in part unsuccessful as the method was only able to detect cells that were attached directly to the surface of the coupons as opposed to being embedded in biofilm structures [1]. Although the PNA probe is well-known for being able to penetrate in biofilm structures due to its hydrophobic character [5], in the current study it also failed to totally penetrate thicker biofilms. Moreover, it was observed that more mature biofilms have a very bright EPS

(exopolymeric substances) matrix that interferes with the observations (Figure 3.3a and c). This problem was overcome by resuspending the biofilm, diluting the sample and filtering onto a membrane for FISH quantification (Figure 3.3b).

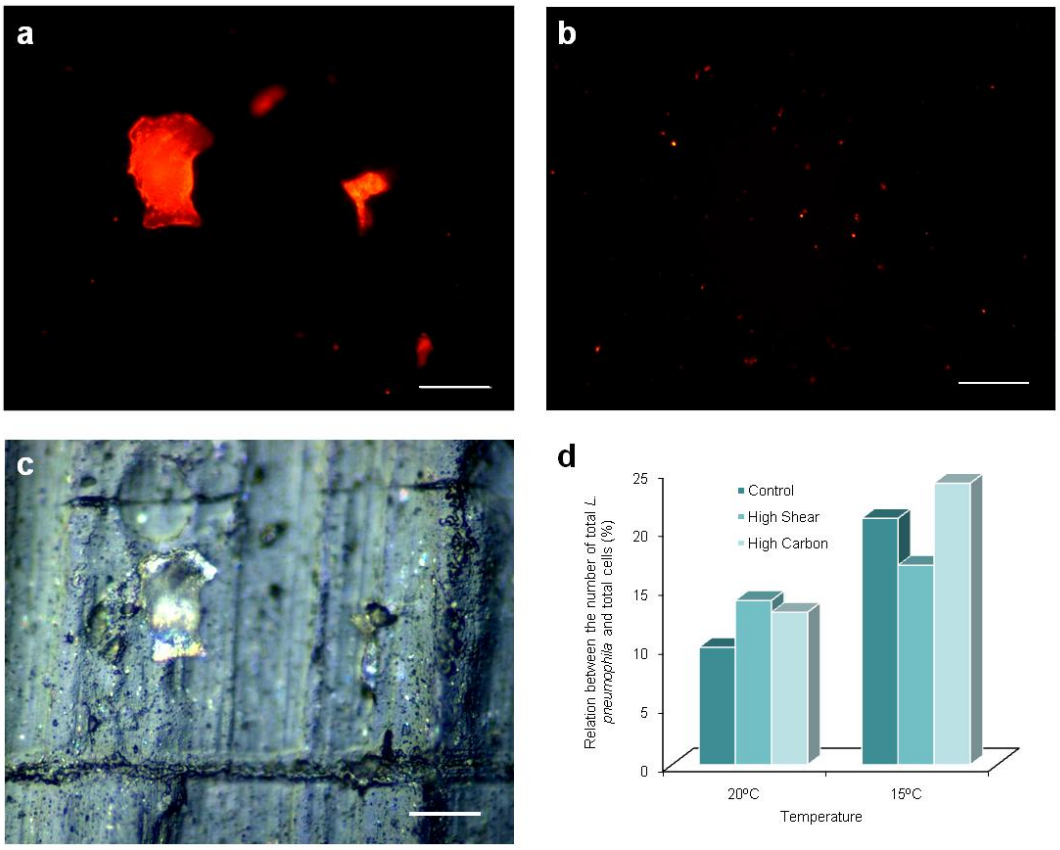

Figura 3.3 Microphotograph of hybridization with the *L. pneumophila* specific PNA probe: uPVC coupon visualized under the TRITC channel (a) and scraped cells homogenized and filtered onto a membrane visualized under the EDIC channel (c). Bars represent 20 μm. Average of the percentage of total *L. pneumophila* and total flora (PNA/SYTO9), for all the conditions tested at both temperatures (d).

In Figure 3.3d it is possible to observe some significant differences which illustrate the influence of the different conditions on the inclusion of *L. pneumophila* in heterotrophic biofilms according the conditions that the biofilm was formed. The relation of *L. pneumophila* was on average 10% at 20ºC of the total biofilm microbial consortium in the control experiment and did not change significantly with the different conditions tested (P>0.05). However when temperature is decreased from 20ºC to 15ºC the percentage of total *L. pneumophila* in relation to total cells is double for the control and high carbon concentration but similar for high shear stress (P=1.000).

3.3.4 Confirmative tests and bacterial identification

In a preliminary experiment the biofilm samples were plated on BCYE and GVPC agar plates. The microorganisms failed to grow on GVPC most of the time and the few colonies obtained did not have a typical *L. pneumophila* physiology. It was also observed that the microorganisms growing on GVPC were also growing on BCYE. All microorganisms grown on BCYE were isolated and a series of *L. pneumophila* confirmative tests were performed [9]. All strains grew on GVPC but failed to grow after acid treatment except one (Table 3.2).

Table 3.2 Results of 16S DNA sequencing and PNA test for the colonies isolated on BCYE and R2A and confirmative tests performed on colonies isolated on BCYE and on *L. pneumophila* NCTC12821.

Medium/ Physiology	Identification by 16S DNA sequencing	PNA	Before treatment			After acid treatment			After heat treatment		
			BCYE	BCYE -cys	GVPC	BCYE	BCYE -cys	GVPC	BCYE	BCYE -cys	GVPC
R2A/white	Acidovorax spp.	-									
R2A/yellow	Sphingobium yanoikuyae	-									
R2A/orange	Saprospiraceae spp.	-									
BCYE/purple	*Stenotrophomonas* spp.	-	+	+	+	-	-	-	+	+	+
BCYE/pink	*Pseudomonas* spp.	nd	+	-	+	-	-	-	+	+	-
BCYE/white	*Mycobacterium chelonae*	-	+	-	+	+	+	+	+	+	+
BCYE/yellow	*Variovorax paradoxus*	-	nd	nd	nd	nd	nd	nd	nd	nd	nd
L. pneumophila	n.a.	+	+	-	+	+	-	+	+	-	+

Legend: na: not applicable; nd: not determined; -: negative result; +: positive result

The conjugation of all the results indicated that none of the microorganisms isolated were *L. pneumophila* species, what was confirmed by the results of the 16S DNA sequencing that identified them as *Mycobacterium chelonae*, *Pseudomonas* spp., *Variovorax paradoxus* and *Stenotrophomonas* spp. (Table 3.2).

All of the microorganisms isolated on BCYE and R2A were also tested with the highly specific *L. pneumophila* PNA probe and results showed that these microorganisms did not bind with the PNA probe (Table 3.2), confirming the results obtained in a previous study [27].

3.4 Discussion

The fresh medium flow rate that was feeding the biofilm-growing vessels was chosen based on the literature [1, 21, 22]. By maintaining a dilution rate of 0.2 h^{-1}, equivalent to a mean generation time of 3.5 hours, a washout is promoted and cells have little time to grow in the planktonic phase, creating the typical conditions to form biofilms [12]. The similarity in the numbers obtained for total planktonic cells in all the chemostats at both temperatures confirms that there is little growth in the planktonic phase, the differences being due to biofilm detachment. Apparently at 20ºC the shear stress does not influence the cultivability of HPC, as the average numbers were quite similar to the control experiment contrary of what happens at 15ºC where a decrease was observed in the cultivability numbers when shear stress was higher ($P<0.05$). When carbon was supplemented the number of total cells was significantly higher compared to the other two chemostats and the cells also appeared much more active, as cultivable numbers were also higher ($P<0.05$). This might be due the sloughing off of larger portions of biofilm as it was noticed that the biofilms grown under high carbon concentration were thicker and have a slimier aspect, due the formation of the EPS matrix.

In Figure 3.2a and b it is possible to confirm that, at 20ºC and 15ºC, respectively, the biofilm formation kinetics follows a typical curve described by other authors [2, 21, 22]. In spite of the fact that the number of total cells are not statistically different for the three conditions tested for the same temperature ($P>0.05$), when the cultivability is studied the influence of the different conditions is noticeable ($P<0.05$). This comparison leads to a conclusion that the carbon concentration influences the biofilm growth more than the shear stress, probably stimulating the growth and multiplication of at least some microorganisms inside of the biofilm. This is corroborated by the fact that, at 20ºC, the total cells of the biofilm grown under high carbon concentration increased faster with biofilm age, contrary to what happens in the other two fermenters. At 15ºC the increase is smoother. This indicates that the biofilm behavior of this potable water community is greatly influenced by temperature, which is corroborated by the fact that at 15ºC there are more cells in the biofilm than at 20ºC ($P<0.05$).

At 20ºC it is observed that the percentage of total *L. pneumophila* in relation to total cells does not vary significantly with shear stress and carbon concentration, indicating that for the experimental conditions tested there is no selection of total *L. pneumophila* adhered to the uPVC surfaces. Conversely, at 15ºC, although the differences are not very evident ($P>0.05$), this percentage diminishes slightly when the shear stress is high. Comparing the percentage obtained at 15ºC and at 20ºC it is possible to conclude that lower temperatures favour the inclusion of *L. pneumophila* during biofilm formation, especially in the control and when carbon is added.

Concerning the temperature effect on biofilm formation, it was observed that at 15ºC the number of total cells and total *L. pneumophila* is higher, showing that at lower temperatures adhesion is promoted, which is perhaps not surprising as at this temperature cells should be more stressed. It should be noted that the optimal growth temperature of this opportunistic human pathogen might exceed 37ºC under some circumstances [16].

The lack of recovery of cultivable *L. pneumophila* can be due the overgrowth of other microorganisms but also by the ability that this microorganism has shown to enter into a VBNC state when stressed. However, it has also been demonstrated that in favourable conditions this microorganism is able to recover cultivability and cause infections [6, 8]. The PNA-FISH method is a tool that can be successfully used to detect specific pathogens in drinking biofilms [1, 17, 27]. Previous studies demonstrated that the PNA probe PLPNE620 is highly specific for *L. pneumophila* and do not bind with other drinking water microorganisms inside of biofilms unless the biofilm has been spiked with *L. pneumophila* [27].

In this study, it has been shown that even having high numbers of total *L. pneumophila* present the recovery of this pathogen to artificial media might remain elusive. It was also observed that the signal emitted by the cells labeled by PNA-FISH remained bright, which is indicative of a high rRNA content and therefore of cell viability [1, 17, 27]. This points to the existence of VBNC cells that, not being detected by standard methods, can give false results concerning water quality. This has public health concerns as Steinert and colleagues [24] have hypothesized that Pontiac fever is caused by VBNC *L. pneumophila*. Moreover, if treatment of water contaminated with VBNC *L. pneumophila* was not improved, then this could lead to resuscitation and multiplication of the pathogen under appropriate conditions such as in biofilms in static dead ends of water supply pipes or in shower heads or in the sediment of corroding water tanks. This effect would be exacerbated if the carbon concentration of these waters was higher than for the normal water supply, increasing the numbers of VBNC and/or cultivable *L. pneumophila* present.

The fact that the percentage of *L. pneumophila* was considerably high even in unfavourable conditions (on average 21% of the total microbial community in the control vessel), raises new concerns about the presence of *L. pneumophila* in drinking water systems and even though outbreaks of Legionnaire's disease are not that frequent, it is possible that infections caused by this pathogen still happen in a mild form, and are being ignored.

3.5 Acknowledgments

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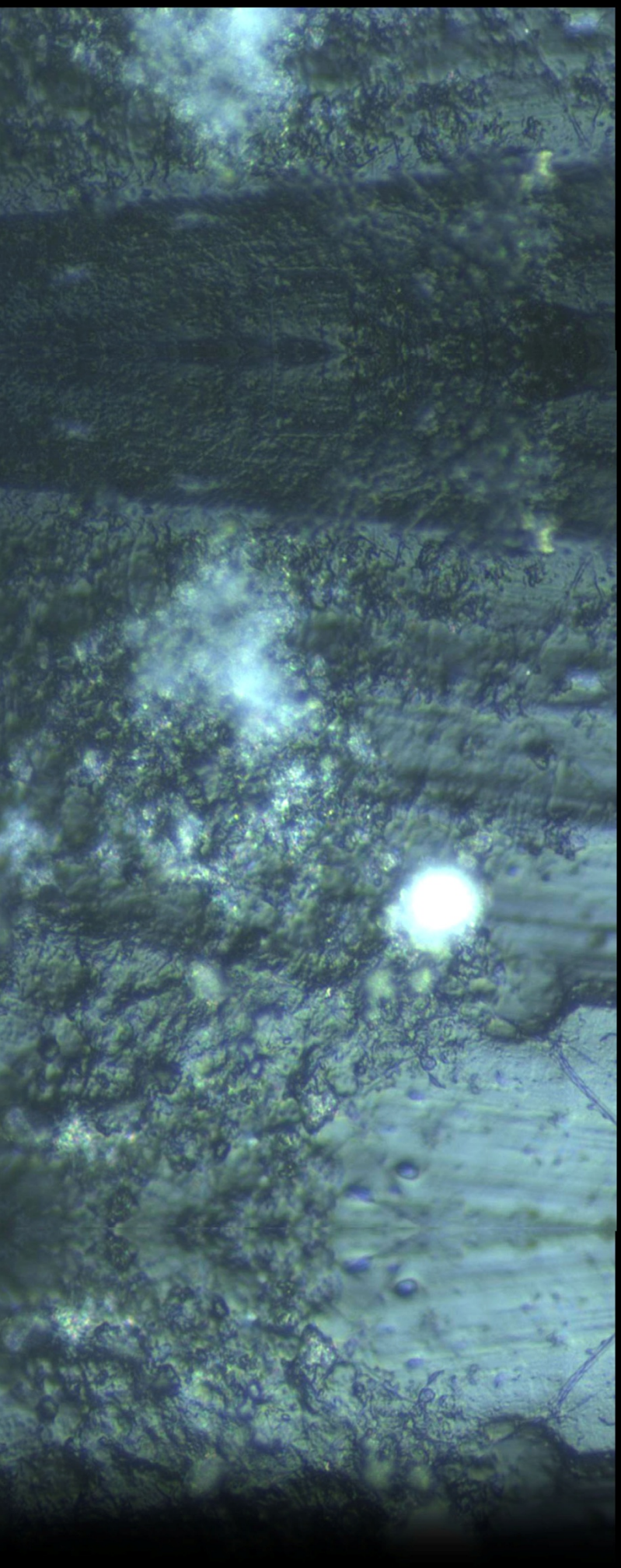

Incorporation of natural uncultivable *Legionella pneumophila* into potable water biofilms provides a protective niche against chlorination stress

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Legionella pneumophila is a waterborne pathogen which has been sporadically isolated from drinking water distribution systems (DWDS). Resistance to disinfectants is mainly attributed to their association with amoebae but biofilms are also thought to provide some degree of protection. In the present work, a two-stage chemostat was used to form heterotrophic drinking water biofilms and study the influence of chlorine concentration on the presence of naturally-occurring *L. pneumophila*. The pathogen was tracked in planktonic and sessile biofilms phases using standard culture recovery techniques for cultivable cells and a pepide nucleic acid fluorescence in situ hybridization assay for total cells. Results showed that total numbers of *L. pneumophila* in biofilms are not affected by the different concentrations of chlorine, although cultivable *L. pneumophila* could not be recovered. Efficient prevention of outbreaks from this bacterium must therefore concentrate on preventing *L. pneumophila* from re-entering an infectious state by maintaining residual disinfectant levels through the entire DWDS network in order to restrict their resuscitation via contact with amoebae.

4.1 Introduction

Under stressful conditions microorganisms can adhere to surfaces to form a biofilm, where they are protected from external stressors, such as the action of biocides [8, 10, 11, 19, 21]. Consequently, it is no surprise that biofilms are ubiquitous in drinking water distribution systems (DWDS) and, in some cases, are related to public health problems [8, 32]. Health concerns arise not only due to the protection that biofilms can offer to some pathogens, but also to an increased resistance to biocides being developed by pathogens as an adaptation mechanism. This resistance has been observed for chlorine, the disinfectant most commonly used in DWDS disinfection [29].

Disinfection efficiency is still assessed by standard cultivation methods, however it is well known that some microorganisms can enter a viable but non-cultivable (VBNC) state, which means that they are not able to grow on highly nutritious and selective artificial media but still retain viability and recover their activity when favourable conditions are found [14, 15, 26, 34]. The non-cultivability of fastidious microorganisms requires the development of new techniques to replace standard methods [36]. Recently, molecular techniques to detect specific pathogens in biofilms have been developed, most noticeably the combination of fluorescence *in situ* hybridization with the use of peptide nucleic acid probes (PNA-FISH) [4, 22, 35].

Legionella pneumophila is one of the pathogens most commonly isolated from DWDS [3, 18]. The natural habitat of this microorganism is considered to be freshwater and it is believed that a parasitic relationship with other environmental microorganisms, such as amoebae, is necessary for *L. pneumophila* to multiply [1, 24, 30]. The ability of this pathogen to incorporate into biofilms has been well-documented [27, 28], however these studies used type collections strains to spike the chemostats and also relied on standard plating procedures for quantification, which fail to detect VBNC cells. In fact, it has been demonstrated that ubiquitous uncultivable *L. pneumophila* can be detected in water by PCR [9, 20, 38]. To understand the real numbers at which *L. pneumophila* might reside in DWDS biofilms and how chlorine concentration affects these numbers is of utmost importance, as it has been recently published that resuscitation of VBNC *L. pneumophila* by contact with amoebic trophozoites occurs even after contact with sodium hypochlorite-disinfected water sources [5, 12].

As such, the aim of this work is to study the effect of different concentrations of chlorine on *L. pneumophila* associated with drinking water biofilms, and compare the numbers obtained by standard culture methods with the numbers obtained with PNA-FISH when a PNA probe highly specific for *L. pneumophila* is used.

4.2 Material and Methods

4.2.1 Biofilm experiments

The formation of biofilms was carried out using a two-stage chemostat model system [17]. The first stage consisted of one 1-litre vessel (seed vessel) and the second stage consisted of three 1-litre vessels running in parallel, but connected in series with the seed vessel. All chemostats were autoclaved and filled with filter-sterilized tap water (1-litre). The seed vessel was then inoculated with a microbial consortium that was obtained from tap water by filtration through a 0.2 μm pore size Nylon filter (Pall Gelman, UK). Preliminary experiments have shown that there was autochthonous *L. pneumophila* in the chemostats, by using the specific *L. pneumophila* PNA probe. The seed vessel was maintained in batch mode for two days to promote microbial growth and then changed to a continuous mode, being fed with filter-sterilized and dechlorinated tap water at a flow rate of 50 ml h^{-1}. This chemostat was operated at room temperature and stirred at 300 rpm to ensure that the oxygen and nutrient concentration were homogeneous. The exit culture was divided in three and used to feed the second stage chemostats; the biofilm-growing vessels. Each biofilm-growing vessel was also fed with fresh media (filter-sterilized tap water) at a flow rate that maintained the dilution rate at 0.2 h^{-1} to promote the typical environmental conditions for biofilm growth. All the vessels were stirred at 300 rpm and the temperature was controlled at 15ºC by a proportional integral derivative unit system (Brighton Systems, UK). To one of the biofilm-growing vessels no chlorine was added, serving as a control, and to the other two vessels chlorine was continuously supplemented, with the concentration maintained at 0.2 mg l^{-1} and 1.2 mg l^{-1}. After 10 days, conditions in the biofilm-growing vessels were stable and sterile unplasticized polyvinylchloride (uPVC) coupons could be immersed. The coupons were removed after 1, 2, 4, 8, 16 and 32 days, gently rinsed to remove planktonic cells attached to the surface of the biofilm, and scraped to quantify sessile cells.

4.2.2 Treatment of coupons

In this study 1 cm^2 uPVC coupons were used as a surface to grow biofilms. Coupons were immersed in water and detergent for 5 minutes, washed with a bottle brusher, rinsed twice in distilled water and air-dried. Subsequently, they were washed in 70% (v/v) ethanol to remove any organic residue that could be on their surface, suspended on a titanium wire and autoclaved [17].

4.2.3 Quantification of planktonic cells

At day 0 (when coupons were immersed) and at all sampling days, water samples from the seed and biofilm-growing vessels were also taken for total cells, heterotrophic cells (HPC) and cultivable *L. pneumophila* quantification. Total cells were quantified by SYTO 9 (Molecular Probes, Invitrogen, UK). In summary, 1 ml of an appropriate dilution was mixed with 0.5 μl of SYTO 9, incubated in the dark for 15 minutes, filtered through a 0.2 μm pore size black polycarbonate Nucleopore® membrane

(Whatman, UK) and allowed to air-dry. Then, a drop of non-fluorescence immersion oil (Fluka, UK) and a coverslip were added before observation under a Nikon Eclipse E800 episcopic differential interference contrast/epifluorescence (EDIC/EF) microscope (Best Scientific, UK) [16]. As the cells were homogenously distributed, several fields were chosen at random and counted on each membrane. Heterotrophic species were quantified by heterotrophic plate count (HPC) on low nutrient R2A medium (Oxoid, UK) and incubated at 22ºC for 7 days. Cultivable *L. pneumophila* were quantified by plating onto Buffered Charcoal Yeast Extract (BCYE) agar plates (Oxoid, UK) and incubated at 30ºC for up to 14 days.

4.2.4 Quantification of sessile cells

Coupons were immersed in 2 ml of filter-sterilized tap water containing sterile 2 mm diameter glass beads (Merck, UK) and vortexed for one minute to remove all the biofilm from the coupons surface and homogenize the suspension. Total cells, HPC, and cultivable *L. pneumophila* were quantified using the methods described above. In addition total *L. pneumophila* were quantified using the highly specific PNA probe with the following sequence 5'-CTG ACC GTC CCA GGT-3' (PLPNE620) (Eurogentec, Belgium) [35]. In that case, 1 ml of an appropriate dilution was filtered through a 0.2 μm anodisc membrane (Whatman, UK) and air dried. Then the membrane was covered with 90% (v/v) ethanol to fix the cells and air dried. The hybridization, washing and microscopy observation method were performed as described by Wilks and Keevil [35].

4.2.5 Chlorine measurements and inactivation

Two of the biofilm-growing vessels were also continuously fed with chlorine, provided by 2 working solutions prepared every two days from a 5 g l^{-1} stock solution (Guest Medical, UK). Chlorine concentration was controlled on a daily basis by measuring free chlorine in the water vessels by the N,N-dimethyl-p-phenilenediamine (DPD) colorimetric method, as described in the Standard Methods for the Examination of Water and Wastewater [2], except for the wavelength used (492 nm). To quantify planktonic and sessile cells from these two vessels, it was necessary to neutralize chlorine. For that sodium thiosulphate (Sigma, UK) at a final concentration of 5 mg l^{-1} was added to the water samples and to the water where the coupons were immersed.

4.2.6 Statistical analysis

The homogeneity of variances of total number of cells, total *L. pneumophila*, HPC and relation between *L. pneumophila* of cells and total cells was checked by the Levene test for equality of variances using a statistical package (SPSS Inc., Chicago IL, USA). Differences were subsequently compared by a one-way ANOVA followed by a Bonferroni post hoc test. Differences were considered relevant if $P<0.05$.

4.3 Results and Discussion

4.3.1 Population in the planktonic phase

After 10 days operating in a continuous mode, the microorganisms present in the inoculum obtained from tap water were already adapted to the chemostat environment and the experiment was started by immersing the uPVC coupons in the biofilm-growing chemostats. As such, the numbers of total cells in the planktonic phase, in general, were not statistically different ($P>0.05$), being the average of total numbers for all the chemostats summarised on Table 4.1. Comparing the results obtained for the different concentrations of chlorine added, it was observed that the HPC numbers decreased significantly with the increase of chlorine concentration ($P<0.05$); thus the concentration of cells was almost 1-log lower in the chlorinated vessels. The surviving culturable HPC represented only 5% of the total cells present when exposed to 1.2 mg Cl_2 l^{-1} compared to almost 40% in the absence of chlorine. In terms of total cells there were significant differences between the control and the chlorinated conditions ($P<0.05$) but the results were similar when the two concentrations of chlorine are compared ($P>0.95$). This indicates that different concentrations of chlorine (at least for low concentrations) have little effect on total numbers of cells but they become less cultivable.

Table 4.1 Numbers of planktonic total cells, HPC Numbers of planktonic total cells, HPC and relation between HPC and total cells in the seed, control and in the chlorinated biofilm-growing vessels.

	Total cells x 10^{-6} (cells ml^{-1})	**HPC x 10^{-5} (CFU ml^{-1})**	**HPC / total cells (%)**
Seed vessel	2.46	5.73	23.3
Biofilm-growing vessels			
0.0 mgCl_2 l^{-1}	2.10	8.38	39.9
0.2 mgCl_2 l^{-1}	1.26	1.98	15.7
1.2 mgCl_2 l^{-1}	1.55	0.78	5.0

The quantification of cultivable *L. pneumophila* was not possible for any sample from the seed vessel and the biofilm-growing. This occurred mainly because the two-stage chemostat system was not spiked with a strain from culture collections, but found instead to be colonized by environmental *L. pneumophila* as assessed by the highly specific PNA probe. It is well-known, however, that many

environmental strains of *L. pneumophila* are typically VBNC and as such unable to be recovered to plating media from water [9, 20, 38]. In addition, other microorganisms were able to grow on the BCYE medium, which means that even if a small proportion of *L. pneumophila* cells are viable, these might be overgrown by other species (see also Chapter 3).

4.3.2 *L. pneumophila* in heterotrophic biofilms

In Figure 4.1, the curves representing the numbers of cells in the biofilm (total cells, total *L. pneumophila* and HPC) denote the traditional shape of biofilm accumulation [7, 27, 28]. It is possible to observe that most of the total cells and total *L. pneumophila* (calculated using the *L. pneumophila* specific PNA probe) attached during the first few days and then varied within a narrow range (P>0.05).

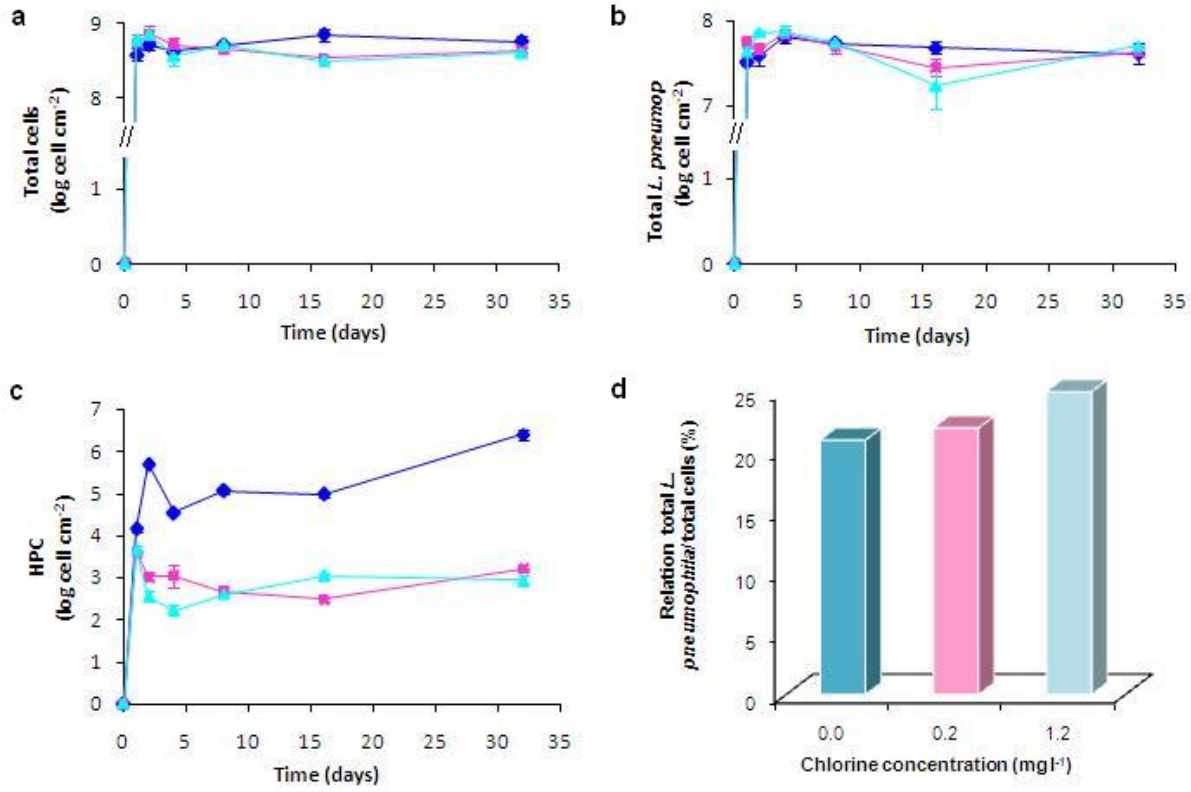

Figura 4.1 Variation in the total cell number (a), total numbers of *L. pneumophila* (b) and HPC (c) in biofilms formed when no chlorine is added (◆), when chlorine is continuously added to a final concentration of 0.2 mg l^{-1} (■) and 1.2 mg l^{-1} (▲). Average of the relation between the numbers total *L. pneumophila* and total cells for all the conditions tested (d).

Figure 4.1 shows that the numbers of total cells in the control vessel were only slightly lower than the numbers obtained when chlorine was added. Biofilms are preferentially formed by stressed cells and

as chlorine is a well-known stressing agent it comes as no surprise that in the vessels where chlorine is added more cells attach to the surface to form the biofilm. This is corroborated by the direct observation of the uPVC coupons using episcopic differential interference contrast (EDIC) microscopy, showing that the biofilm formed under chlorinated conditions was quantitatively and structurally similar to the biofilm formed when no chorine was added (Figure 4.2), although coupons removed from the non-chlorinated vessel looked slimier than the coupons with biofilm growth under chlorine addition. Moreover, several studies have shown previously that biofilms can even grow in the presence of chlorine [19, 25].

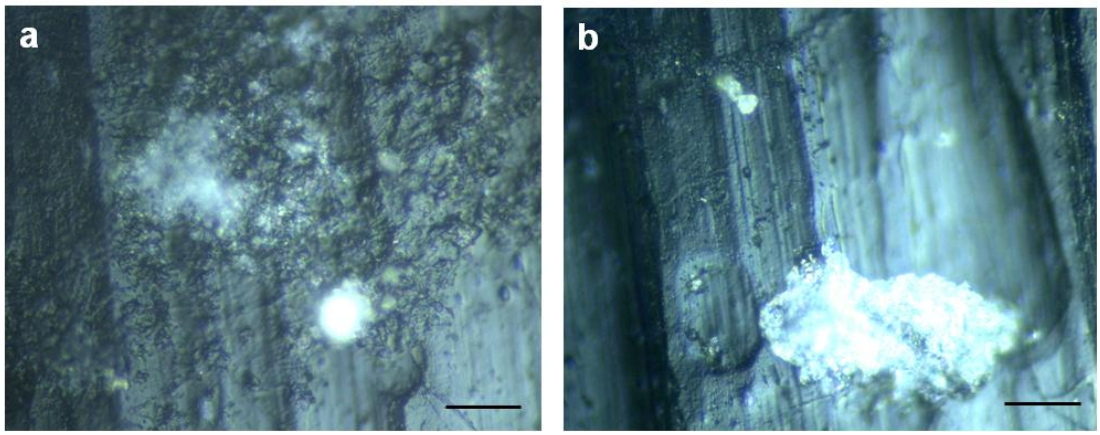

Figura 4.2 Microphotograph of a uPVC coupon visualized under the EDIC channel. The coupon was covered with a 32 days-old biofilm formed in the absence of chlorine (a); in the presence of 1.2 mg ml^{-1} of free chlorine (b). Bars represent 20 μm.

The numbers of total *L. pneumophila* quantified by the use of the PLPNE620 PNA probe did not vary significantly with time for all the concentrations tested ($P>0.05$) and were similar appear to be similar on biofilms formed under the three different concentrations. This suggests that chlorine has also little effect on this particular pathogen to incorporate in biofilms. On the other hand, the percentage of total *L. pneumophila* was on average approximately 20% of the total cells, under all biofilm formation conditions. This is still a strikingly high result considering that the chemostats were not spiked with *L. pneumophila*. It is true that there is no certainty on whether *L. pneumophila* is still in the viable state, as no cultivable *L. pneumophila* was successfully recovered from any of the biofilm samples, but the fact that the PNA probe is able to detect it and exhibit a bright fluorescence signal implies the presence of a high 16S rRNA within the cells which strongly suggests that the bacterium might be in a VBNC state. The non-recovery of cultivable *L. pneumophila* had also been observed in a previous study investigating nutrient deprivation and the microorganisms isolated on selective agar were identified afterwards as not being *Legionella* species by 16S DNA sequencing (Chapter 3).

The major differences appear when cultivable cells were quantified, as HPC numbers are much lower in chlorinated conditions comparing to the control assay ($P<0.05$), which is supported by two studies conducted by Codony *et al*. [6, 7], where it is verified that under chlorinated conditions the total

number of cells are similar to the control study but cells are less cultivable. In fact it was noticed that in the control the HPC numbers were 2-log lower than in the biofilms formed in chlorinated water while the difference for the two concentrations used was not significant (P>0.05), indicating that chlorine maintains biofilm formation within a certain range.

4.3.3 Impact of chlorine on biofilm physiology

The morphology of the colonies obtained on R2A and on BCYE was similar for the samples of planktonic and sessile cells, although in some sporadic cases the colonies appeared smaller and less mucoid in the samples obtained from the chlorinated vessels. On the other hand, the planktonic cells stained with SYTO 9 and observed by microscopy had mainly a rod shape and were very bright while the sessile cells appeared as small and faint dots (results not shown) which is not surprising as cells in a biofilm normally adapt and change their characteristics [13, 33]. It was also observed that the coupons removed from the control vessel started to have a slimy aspect after 2 weeks but the coupons removed from the chlorinated vessels never showed a slimy appearance. This slime might be due to the presence of polymeric substances that form the typical EPS (extracellular polymeric substance) matrix that involves the biofilms, protecting them from the external conditions [31, 36]. Under chlorinated conditions there was no visible EPS which might mean that the cells in the biofilm are more exposed to chlorine, as the diffusion through the biofilm with scarce EPS matrix is easier and deeper than when the matrix is thicker, resulting in loss of cultivability when biofilms are formed in chlorinated water.

4.3.4 Impact of this study on public heath

This work reveals some important behaviour and characteristics of environmental *L. pneumophila*, showing that this microorganism can reach up to 25% of the total microbial community of a biofilm, despite the presence of chlorine.

The highest concentration of chlorine used in this work was greater than the concentration advised by the World Health Organization (between 0.2 and 0.5 mg l^{-1}) [37] but the numbers of total *L. pneumophila* remained constant. Although no cultivable *L. pneumophila* was detected, considering that cells detected by PNA FISH (cells with intact rRNA) might be still viable and that previous studies have shown that *L. pneumophila* can lose cultivability without losing viability (Chapter 2), a pertinent question remains: is *L. pneumophila* retaining its virulence and still able to cause infections? Relying on the studies conducted by Hussong and colleagues [14] that showed that this pathogen can lose cultivability in stressful conditions but maintain its virulence then it is plausible that the answer is yes.

As it has been stated before, *L. pneumophila* total cell numbers in biofilms were independent of chlorine concentration, which supports the view that efforts to eliminate the pathogen from DWDS are inadequate. In fact, if chlorine concentrations peak at a certain point in the DWDS, thereby contributing for the enrichment of the pathogen in biofilms, *L. pneumophila* might use their dominant

position in terms of numbers and viability to further establish itself downstream in the DWDS. As such, a better strategy would be to make sure that residual disinfectant levels through the entire DWDS network are maintained in order to restrain *L. pneumophila* resuscitation via contact with amoebae.

With the constant disinfection failures that have been observed in many DWDS, it is perhaps surprising that *L. pneumophila* infection has not been even more widespread. Possible explanations include stress-induced lack of virulence, the minimum inoculum concentration to establish infection and the effect of low temperatures [23], the fact that most infections occur via aerosolized particles and even the possible lack of infectious ability by many strains due to their genetic repertoire. Whatever the real answer is, it appears that biofilms play a key role in sustaining the survival of the bacterium under stressful conditions, so that cells can replicate again as soon as more favorable conditions are found.

4.4 Acknowledgements

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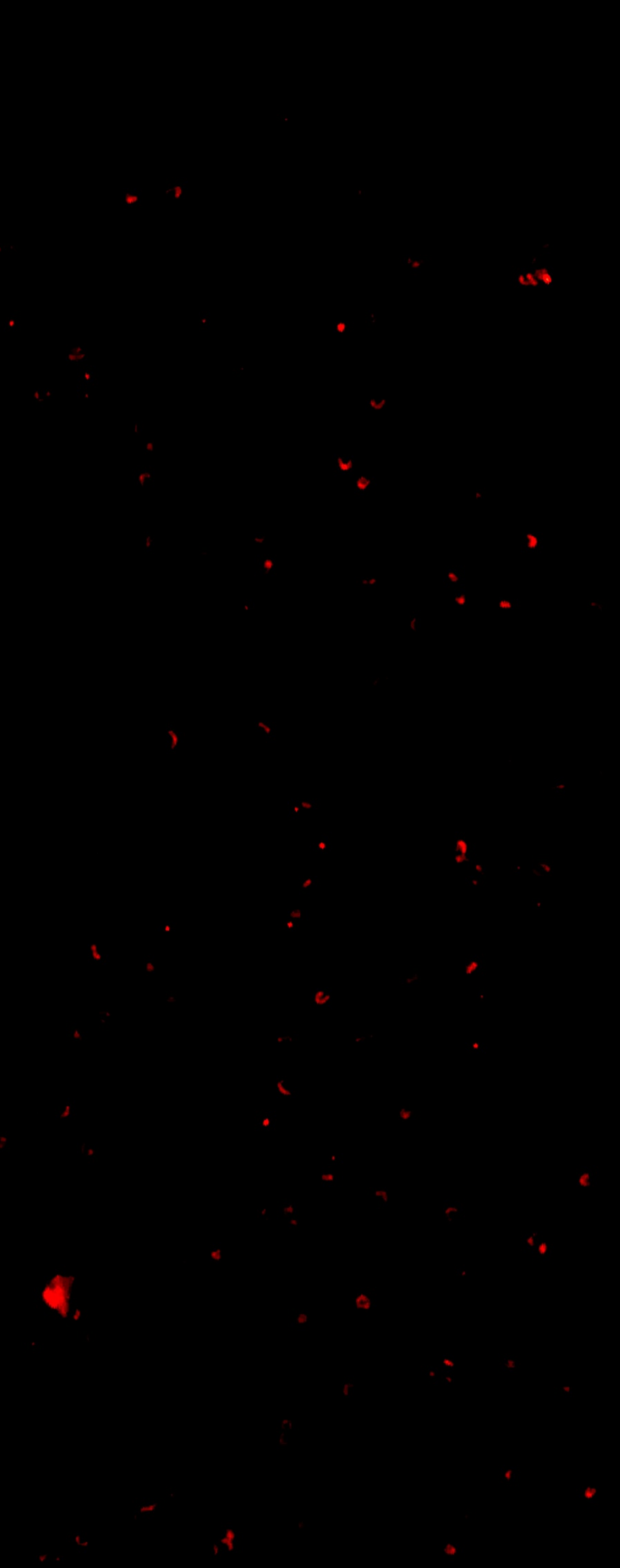

Persistence of *Helicobacter pylori* in heterotrophic drinking water biofilms

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N. F. Azevedo
S. A. Wilks
M. J. Vieira
C. W. Keevil

Although the route of transmission of *Helicobacter pylori* remains unknown, drinking water has been considered as a possible vector of transmission. In water, it has been previously shown that biofilms act as a protective niche to several pathogens, protecting them from stressful conditions, such as low carbon concentration, shear stress and less-than-optimal temperatures. In this work, the influence of these three parameters on the persistence and cultivability of *H. pylori* in drinking water biofilms has been studied. Autochthonous biofilm consortia were formed in a two-stage chemostat system and then inoculated with the pathogen. *H. pylori* total cell numbers were quantified by microscopy using a specific *H. pylori* 16S rRNA peptide nucleic acid probe whereas cultivable cells were assessed by standard plating onto the selective *Helicobacter pylori* (HP) medium. Cultivable *H. pylori* could not be detected at any time point, but the ability of *H. pylori* cells to incorporate, undergo morphological transformations, persist and even agglomerate in biofilms for at least 31 days without a noticeable decrease in total cell numbers - between, on average, 1.54×10^6 and 2.25×10^6 cells cm^{-2} - or in intracellular rRNA content may indicate that this loss of cultivability is due to the entry into a VBNC state. Unlike previous results obtained for pure-culture *H. pylori* biofilms, shear stress does not negatively influence the numbers of *H. pylori* cells attached, suggesting an important role for autochthonous aquatic bacteria in retaining this pathogen in the sessile state, possibly by providing suitable microaerophilic environments or linking biomolecules for the pathogen to adhere. Therefore, biofilms appear to provide not only a safe haven for *H. pylori* but a concentration mechanism such that subsequent sloughing releases a concentrated bolus of cells that might be infectious and that would escape routine grab sample microbiological analyses and be a cause of public health concern.

5.1 Introduction

Helicobacter pylori is one of the most prevalent pathogens among human individuals, especially in developing countries where incidence can reach up to 90% of the population [16]. Even though most individuals that are infected by this pathogen are asymptomatic, it is now well established that *H. pylori* can lead to the development of peptic and duodenal ulcer disease and gastric MALT lymphoma [8].

The route of transmission of this pathogen is still unknown. Person-to-person transmission seems most likely as the only place where *H. pylori* has been systematically isolated is the human GI tract [3]. However, some authors suggest that water, food and animals can also be vectors of transmission [3, 7, 10, 17, 20, 29, 38]. The largest obstacle to prove that water is a route of transmission lays in the fact that *H. pylori* has never been cultured from drinking water distribution systems (DWDS) using standard cultivation techniques [3, 19]. Whether this happens due to the fastidious nature of the microorganism or to the loss of viability in water is the key question of the transmission debate. Accordingly, some groups have been attempting to develop artificial media to achieve better culture recovery results than the one obtained with the traditional Columbia blood agar (CBA), such as F-12 [37] or the selective HP medium that has been proposed to recover *H. pylori* from water-exposed, heterotrophic microenvironments [15].

In the meantime, molecular techniques such as polymerase chain reaction (PCR), have demonstrated the presence of *H. pylori* in drinking water distribution systems (DWDS), especially associated with biofilms [10, 29, 30, 40]. This shows that *H. pylori* is present in water but DNA isolation alone fails to provide any indication about the viability of the bacterium.

In recent years another molecular technique, fluorescence *in situ* hybridisation (FISH), has been successfully used to detect this pathogen in DWDS and other water bodies [9, 31]. This technique usually detects rRNA, which implies that it is not only able to detect the presence of *H. pylori*, but also to provide an indication of viability up to a certain extent due to the maintenance of a high rRNA content [6, 28, 42].

The aim of this work was to apply both FISH and a selective culture medium to assess the number of *H. pylori* cells found in autochthonous complex consortia drinking water biofilms (i.e., ubiquitous in tap water biofilms) formed under different conditions in order to better understand the dynamics of *H. pylori* populations in real DWDS.

5.2 Material and Methods

5.2.1 Biofilm experiments

The formation of biofilms was carried out using a two-stage chemostat model system [22]. The first stage consisted of a 1-litre vessel (seed vessel) and the second stage consisted of three 1-litre vessels running in parallel, but connected in series with the seed vessel. All chemostats were autoclaved and filled with filter-sterilized (0.2 μm pore size Nylon filter) tap water (1-litre). The seed vessel was then inoculated with a microbial consortium that was obtained from tap water by filtration through a 0.2 μm pore size Nylon filter (Pall Gelman, UK). This vessel was maintained in batch mode for two days to promote microbial growth and then changed into a continuous mode, being fed with filter-sterilized and dechlorinated tap water at a flow rate of 50 ml h^{-1}. The seed vessel was operated at room temperature (approx. 22ºC) and stirred at 300 rpm to ensure that the oxygen and nutrient concentrations were homogeneous. The effluent was divided in three and used to feed the second stage chemostats; the biofilm-growing vessels. Each biofilm-growing vessel was also fed with fresh medium (filter-sterilized tap water) at a flow rate that maintained a dilution rate of 0.2 h^{-1} to promote typical environmental conditions for biofilm growth. The first vessel was stirred at 300 rpm and with no addition of any carbon source (low shear stress and low carbon concentration – LS/LC), serving as a control. The second vessel was stirred at 1200 rpm but with no carbon addition (high shear stress and low carbon – HS/LC) and the third vessel was stirred at 300 rpm and 8.8 mg l^{-1} of carbon was added by the inclusion of 30 mg l^{-1} of sodium acetate to the fresh medium (low shear stress and high carbon – LS/HC). The temperature was controlled at either 15ºC or 20ºC by a proportional integral derivative unit system (Brighton Systems, UK). After 10 days, conditions and total cell numbers in the biofilm-growing vessels were stable and the biofilm growing vessels were inoculated with *H. pylori* NCTC 11637 at a final concentration of approximately 8 x 10^5 CFU ml^{-1} (determined by measuring absorbance at 640 nm) followed by immersion of sterile polyvinylchloride (PVC) coupons (day 0). The coupons were removed after 1, 2, 4, 8, 16 and 31 days, gently rinsed to remove planktonic cells attached to the surface of the biofilm, and scraped to quantify sessile cells.

5.2.2 Cultivation of *H. pylori*

H. pylori NCTC 11637 was maintained in vials frozen at -80ºC and recovered by plating onto Columbia Blood Agar (CBA) (Oxoid, UK) supplemented with 5% (v/v) defibrinated horse blood (Oxoid, UK) and incubated for 48 hours at 37ºC in a variable atmosphere workstation (MACS VA500, Don Whitley, UK) set to a microaerophilic atmosphere of 10 % CO_2, 7 % H_2 and 3 % O_2; the remainder being N_2. The cultures were subcultured once for 48 hours and used to inoculate the second-stage chemostats.

5.2.3 Preparation of coupons

PVC coupons (1 cm^2) were used as a support for biofilm. PVC was the material chosen for the coupons as it is the material more commonly used in water pipes and also because it has been shown to be one of the less aggressive materials for *H. pylori* survival [2]. Coupons were immersed in water and detergent (Guard professional, UK) for 5 minutes, washed with a bottle brusher, rinsed twice in distilled water and air-dried. Subsequently, they were washed in 70% (v/v) ethanol to remove any organic compounds, attached to the end of a titanium wire and autoclaved [22].

5.2.4 Quantification of planktonic cells

Water samples were taken after 0, 1, 2, 4, 8, 16 and 31 days from the seed and biofilm-growing vessels and were analyzed for total cells, heterotrophic plate count cells (HPC) and cultivable *H. pylori*. Total cells were quantified using SYTO 9 (Molecular Probes, Invitrogen, UK). In short, 1 ml of an appropriate dilution was mixed with 0.5 μl of SYTO 9 (5mM solution in DMSO), incubated in the dark for 15 minutes, filtered through a 0.2 μm pore size polycarbonate black Nucleopore® membrane (Whatman, UK) and allowed to air-dry. Then a drop of non-fluorescence immersion oil (Fluka, UK) and a coverslip were added before observation under a Nikon Eclipse E800 episcopic differential interference contrast/epifluorescence (EDIC/EF) microscope (Best Scientific, UK) [23]. As cells were homogeneously distributed, 10 fields of view were randomly chosen and the number of cells counted on each membrane. HPC were obtained by plating onto R2A medium agar plates (Oxoid, UK) and incubating at 22ºC for 7 days. Cultivable *H. pylori* was quantified by plating in triplicate onto selective HP medium agar plates as described by Degnan et al. [15], using either calf serum or 5% (v/v) defibrinated horse blood (Oxoid, UK) and the addition of 0.5 g l^{-1} pyruvic acid (Sigma, UK). Plates were incubated at 37ºC in a microaerophilic atmosphere for 7 days. The colonies obtained on HP agar plates were tested with the urease test performed according to the manufacturer instructions (Oxoid, UK) and with the specific *H. pylori* peptide nucleic acid (PNA) probe to confirm the identity of *H. pylori* as described below [21].

5.2.5 Quantification of sessile cells

Coupons were immersed in 2 ml of filter-sterilized tap water containing autoclaved 2 mm diameter glass beads (Merck, UK) and vortexed for one minute to remove the biofilm from the coupons' surface and homogenize the suspension. A previous study in which the coupons were observed under EDIC microscopy showed that this method completely removes the biofilm. Total cells, HPC and cultivable *H. pylori* were quantified using the methods described above. In addition, total *H. pylori* were quantified using a specific PNA probe with the following sequence 5'- GAGACTAAGCCCTCC -3' (Eurogentec, Belgium) in a fluorescence *in situ* hybridization assay (PNA-FISH) (20). PNA-FISH was carried out by taking 1 ml of an appropriate dilution and filtering through a 0.2 μm Anodisc membrane (Whatman, UK). This was left to air dry. Then the membrane was covered with 4% (w/v) paraformaldehyde followed by 50% (v/v) ethanol for 10 minutes each to fix the cells and finally air

dried. The hybridization, washing and microscopy observation method was based on the methods described by Guimarães et al. [21]. In this case, 20 fields of view were randomly chosen and the number of cells counted on each membrane.

5.2.6 Identification of sessile cells

All the bacteria isolated onto R2A and HP media were identified by 16S DNA sequencing at DNAVision SA (Belgium). Briefly, for each culture DNA was purified, amplified and sequenced with 16S primers. The analysed fragments were about 1600 base pairs length. The sequenced fragments were then BLASTed in the NCBI public database and therefore obtained the Genbank accession number for the highest sequence similarity value.

5.2.7 Statistical analysis

The homogeneity of variances of total number of cells, total *H. pylori* and HPC and was checked by the Levene test for equality of variances using a statistical package (SPSS Inc., Chicago IL, USA). Differences were subsequently compared by a one-way ANOVA followed by a Bonferroni post hoc test. Differences were considered relevant if $P<0.05$.

5.3 Results and Discussion

5.3.1 Seed vessel

The two-stage chemostat system was left operating in a continuous mode for 10 days to stabilize the microbial consortia in all of the chemostats. After this period of time, the number of total cells and HPC in the seed vessel remained stable during the entire experiment, with an average of 3.23 x 10^6 cells ml^{-1} and 6.83 x 10^5 CFU ml^{-1}, respectively. Autochthonous *H. pylori* were not found in the seed vessel using culture recovery or PNA FISH detection techniques, and so the chemostats from the second stage of the system were inoculated directly with *H. pylori* NCTC 11637, as described above, prior to the immersion of coupons.

5.3.2 Planktonic cells in the biofilm-growing vessels

Initially, the biofilm-growing vessels were maintained at 20ºC to study the influence of shear stress and carbon concentration. In the planktonic phase the increase of shear stress did not significantly influence the total number of cells or HPC (P=1.000), but in the second stage chemostat where acetate carbon was added the HPC numbers increased (P<0.005) (Table 5.1) indicating that growth was carbon limited. However, comparing the percentage of cultivable cells obtained for the three different conditions tested, it is possible to observe that the values are all very similar and within the range of 40-45% of total cells being cultivable. Subsequently, the temperature in the biofilm-growing chemostats was decreased to 15ºC. In the control vessel (LS/LC) the percentage of cultivable cells was very similar to the value obtained at 20ºC (P=1.000), however this value was higher when either shear stress (P<0.005) or carbon concentration (P<0.005) were increased (Table 5.1).

Table 5.1 Average numbers of total cell and HPC in the planktonic phase and relation between HPC and total cell, at 20ºC and 15ºC for all three conditions tested.

	Total cells x 10^{-6} (cells ml^{-1})	**HPC x 10^{-6} (CFU ml^{-1})**	**HPC / total cells (%)**
T = 20ºC			
LS/LC	2.74	1.23	45
HS/LC	2.56	1.01	40
LS/HC	6.99	3.17	45
T = 15ºC			
LS/LC	1.74	0.82	47
HS/LC	1.86	1.18	63
LS/HC	17.8	10.8	61

For all water samples analysed it was not possible to recover cultivable *H. pylori*. Other authors have demonstrated that in pure culture at 15ºC and 20ºC, *H. pylori* is able to maintain cultivability for some days both in a water suspension [1, 4, 34] and in water-exposed biofilms [2, 14], which suggests that the difficulty of recovering cultivable *H. pylori* in this work was due to interactions between this microorganism and other species of the bacterial population present in water, like competition for nutrients or due to the production of toxic compounds by other microorganisms. It is also important to mention that overgrowth of other microorganisms occurred in certain samples. Degnan and colleagues [15] have developed the HP medium as a way to selectively recover *H. pylori* from water and have accordingly challenged the medium with the most commonly-known aquatic microorganisms and with heterotrophic consortia from real water samples, obtaining negative growth in both cases. However, real samples differ from place to place and in the present study, microorganisms were isolated from the chemostat system on HP medium and identified by 16S DNA sequencing as *Brevundimonas* sp., *Mycobacterium chelonae* and *Sphingomonas* spp. (Genbank access number EF194089, AM884326 and AY749436, respectively). None of these microorganisms had been assessed for growth on HP medium in the original experiments by Degnan et al. [15].

5.3.3 Cell population in the biofilm-growing vessels

Figure 5.1a shows the variation of total cells, HPC and total *H. pylori* in biofilms grown at 20ºC for the three conditions tested. As it was obtained for planktonic samples, no cultivable *H. pylori* was recovered on HP media from any of the biofilms samples. The biofilm development follows a kinetic described by other authors, where the adhesion of most cells takes place in the first day, but no statistically significant change occurs afterwards ($P>0.05$) [13, 33]. It should be noted that this pseudo steady-state is actually the result of a dynamic equilibrium typical of biofilms where parts of the biofilm detaching from the coupons are balanced by the adherence of new cells [36, 39]. This same trend was observed for the experiment carried out at 15ºC ($P>0.05$) (Figure 5.1b).

In terms of total numbers of cells at 20ºC, increasing the shear stress did not affect numbers ($P=1.000$), as it was obtained on average 4.31×10^8 cells cm^{-2} when biofilm was formed in the LS/LC vessel and 4.14×10^8 cells cm^{-2} in the HS/LC vessel. However, adding a carbon source caused a two-fold increase in the numbers (1.01×10^9 cells cm^{-2}) even though this result is not statistically significant ($P>0.05$). For HPC numbers the differences were even more evident when the carbon concentration was increased (4.00×10^6 CFU cm^{-2} versus 4.87×10^5 CFU cm^{-2} in the LS/LC and 4.55×10^5 CFU cm^{-2} in the HS/LC ($P<0.1$) which is not surprising as carbon is the limiting nutrient in this aquatic environment. The comparison of the results obtained at both temperatures suggests that when the concentration of carbon is increased the formation of biofilm is favoured at 20ºC ($P<0.005$).

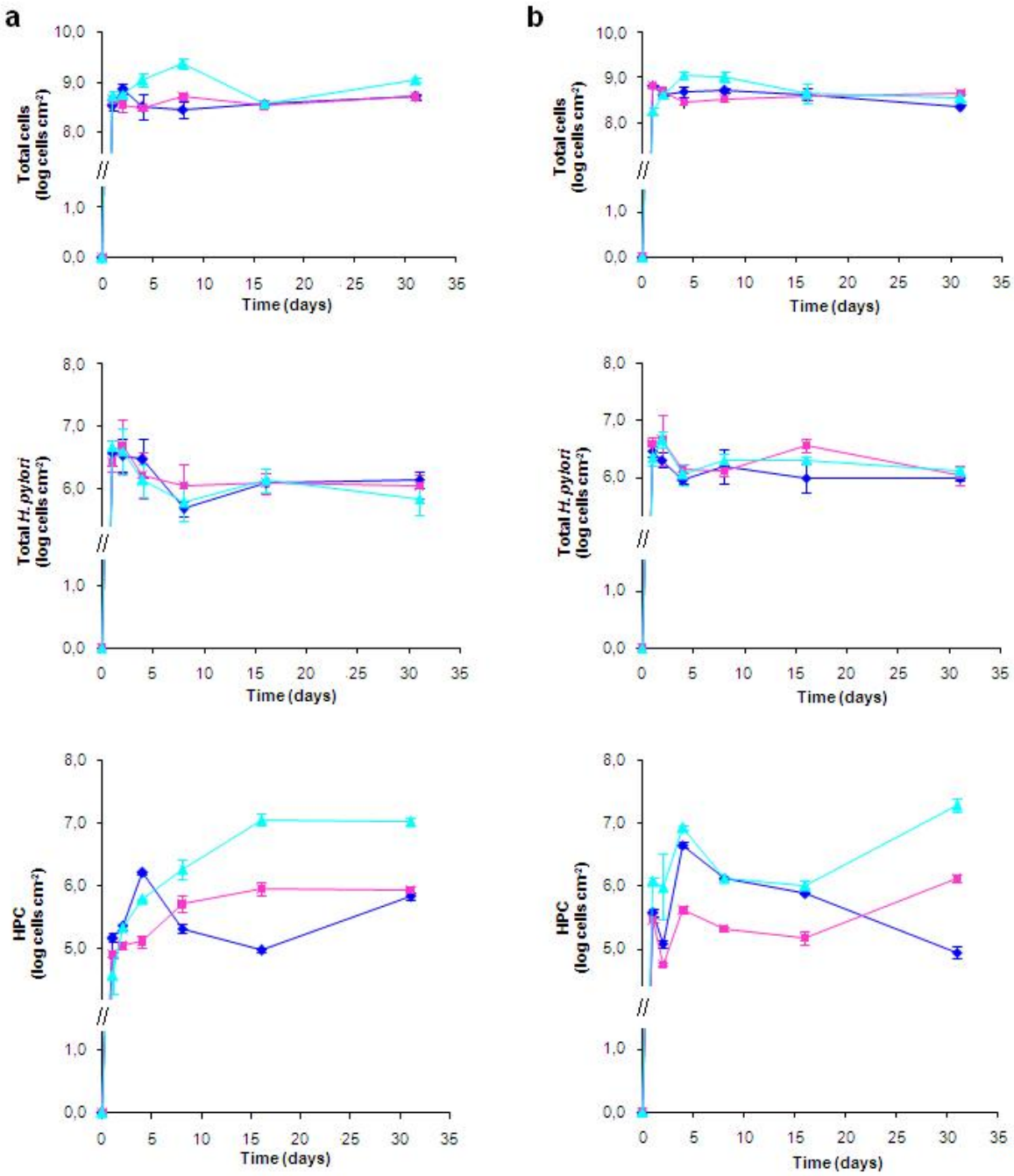

Figura 5.1 Variation in the total cell number, HPC and total numbers of *H. pylori* in biofilms formed at 20ºC (a) and 15ºC (b) under the following conditions: low shear stress and low concentration of carbon (◆), high shear stress and low concentration of carbon (■) and low shear stress and high concentration of carbon (▲). Error bars represent standard deviation.

5.3.4 *H. pylori* total counts in biofilms

One of the most important breakthroughs in this work was to be able to consistently detect and quantify *H. pylori* within biofilm structures by the use of the *H. pylori* PNA probe, as earlier attempts have been hampered by strong autofluorescence of the biofilm stacks in some environments containing autofluorescent contaminants such as polycyclic aromatic hydrocarbons (PAH) [6]. This

issue was overcome by the option of counting the bacteria after removal of the biofilm from the coupons using glass bead agitation and washing. In this way, it is still possible to visualize the matrix structures of biofilm under epifluorescence microscopy (indicated by the large arrows on Figure 5.2a and b), however the autofluorescence is not so bright allowing the observation of the cells. On the other hand, the number of detected bacteria in that earlier study was also considerably lower than the one found here, which demonstrates that most of *H. pylori* cells in the sessile state are embedded within biofilm structures.

When comparing the effect of the different conditions tested, the results obtained for total *H. pylori* (at 20ºC) had a similar trend compared to the results described previously for total cells, as also in this case, the numbers of total *H. pylori* cells were not statistically different (P>0.05) for the three conditions tested (on average 2.25 x 10^6 cells cm^{-2} in the LS/LC, 2.12 x 10^6 cells cm^{-2} in the HS/LC environment and 2.15 x 10^6 cells cm^{-2} in the LS/HC environment) (Figure 5.1a). In relation to the values obtained for total *H. pylori* at 15ºC, there was no significant difference between the three conditions tested (P>0.05), being on average 1.54 x 10^6 cells cm^{-2}, 1.89 x 10^6 cells cm^{-2} and 1.72 x 10^6 cells cm^{-2} in the LS/LC, HS/LC and LS/HC environments, respectively (Figure 5.1b). Comparing the results obtained at both temperatures it is possible to observe that the numbers are also not statistically different (P>0.05) suggesting that the physico-chemical parameters studied in this work did not affect the presence of *H. pylori* in heterotrophic biofilms, indicating that if a pulse (i.e. a sporadic occurrence) of the pathogen passes through a drinking water supply then *H. pylori* will be included in biofilms regardless of the DWDS characteristics.

The lack of effect of temperature on *H. pylori* adhesion has been demonstrated before by Azevedo et al. [5], however, contrary to what was observed in this work, that same study demonstrated that shear stress hinders biofilm formation. Even though the systems used to generate biofilm were different (the system used in the previous study was operated in batch mode, having planktonic *H. pylori* during the entire experiment), this altered behaviour might be more logically explained by the heterotrophic nature of the biofilm in the current work. Heterotrophic DWDS associated-biofilms are known to create a safe haven to protect microorganisms from external stress such as temperature, shear stress, oxygen and nutrient concentration [25, 32, 41], and might help to retain *H. pylori* attached to the surfaces.

In general, the numbers of total *H. pylori* at both temperatures decreased during the first week (P<0.05) and remained more stable afterwards whereas the total number of bacteria in the biofilm remained constant throughout the experiment (Figure 5.1a and b). This might be explained by the fact that the vessels were pulsed with *H. pylori* only at the beginning of the experiment rather than being continually challenged. Consequently, after the initial decline of the pathogen from the chemostats (that occurred in approx. five hours), *H. pylori* cells that were only loosely adhered detached from the biofilm and could not be replenished. After one week, the remaining *H. pylori* cells were already well-protected within the biofilm and for any extra layers of heterotrophic cells that attached on top of them, and hence the number of total *H. pylori* stabilised on most conditions (P=1.000). Figures 5.2a and b

demonstrate that most of *H. pylori* cells in the sessile state are embedded in biofilm structures and sustain this hypothesis.

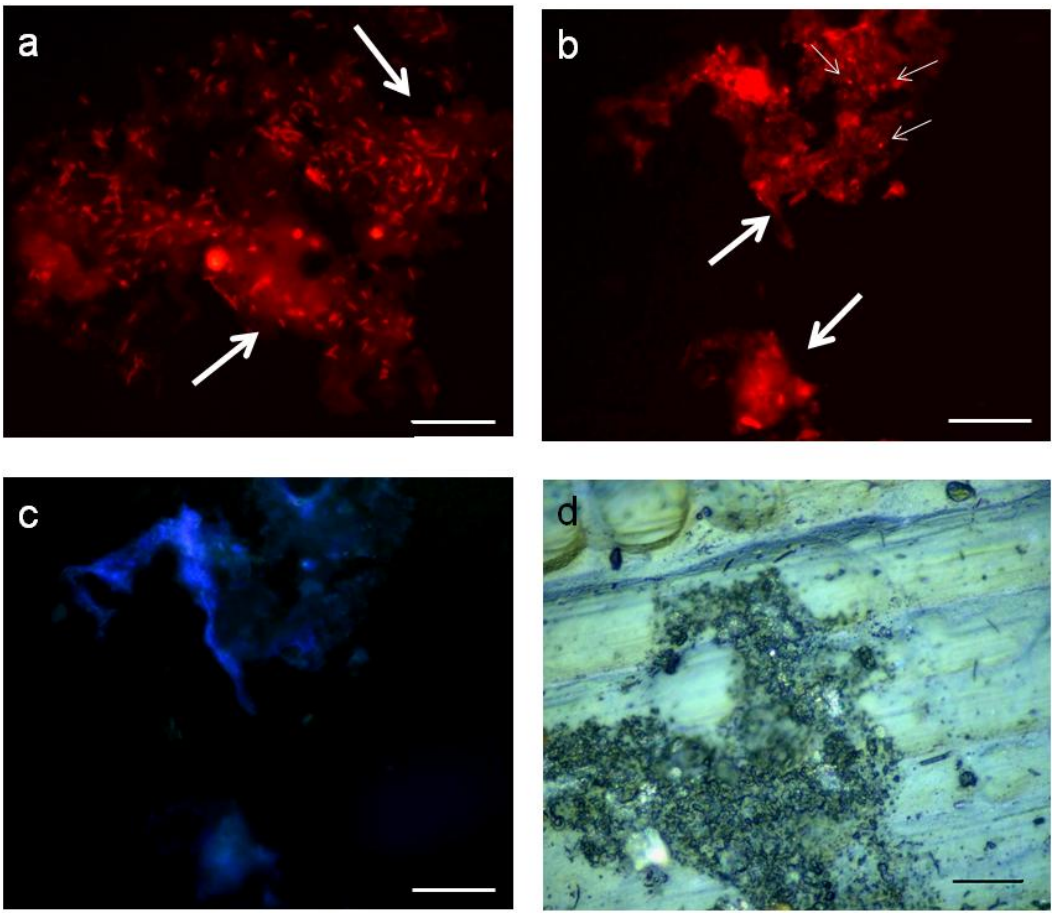

Figura 5.2 Microphotograph of hybridization with the *H. pylori* specific PNA probe in a biofilm grown at 20ºC (a) and at 15ºC (b) using the epifluorescence TRITC filter. Large arrows indicate the autofluorescent matrix of the biofilm whereas thin arrows represent coccoid *H. pylori* embedded in these structures. In (c) the cells were observed using the epifluorescence DAPI filter serving as a control for the autofluorescence of the biofilm stacks and individual cells attached to the substratum. Micrograph of a coupon with a 26 days-old biofilm formed under LS/LC at 15ºC and observed using EDIC microscopy (d). Bars represent 20 μm.

5.3.5 *H. pylori* morphology and location within biofilms

The major difference between the experiments carried out at different temperatures was in the shape of the cells detected by PNA-FISH. Spiral and coccoid-shaped cells were observed at both temperatures; however, at 20ºC there were a larger proportion of spiral-shaped cells than at 15ºC (Figures 5.2a and b). This is extremely relevant as the morphology of *H. pylori* cells has been intimately connected with viability and infection capacity. Although it has been previously shown that coccoid *H. pylori* might correspond to dead cells [18, 27], recent reports on the behaviour of *H. pylori* in water have shown that coccoid cells are the manifestation of an environmentally robust type of cells

that might be understood as being in the viable but non cultivable (VBNC) state [2, 11, 12]. For *H. pylori*, this morphological condition appears to still be able to recover cultivability and cause infection when inoculated in mice [35].

An ecological explanation for the presence of *H. pylori* in biofilms is provided by the microaerophilic nature of this pathogen. In fact, other microorganisms such as *Legionella pneumophila*, *Campylobacter jejuni* and even *E. coli*, have been shown to prefer the microaerophilic environments demonstrated to be present in biofilm stacks [22, 24, 32], due to their intricate structure (Figure 5.2d). A recent study [26] has confirmed that even at high shear stresses, the oxygen concentration remains quite low in fronds or stacks which is certainly beneficial to *H. pylori* and might explain why numbers are not affected at high shear stresses. However oxygen availability is certainly dependent on the conditions under which biofilms are formed and as such it is not yet possible to ascertain that all biofilms contain microaerophilic microniches.

5.3.6 Cultivability of *H. pylori* in water and water-associated biofilms and implications for transmission.

Adams et al. [1] have shown that in pure culture *H. pylori* cells retain cultivability for longer at 15ºC than at 20ºC. In the current study it was not possible to recover cultivable *H. pylori* from water samples and biofilms. However, considering the shape of the cells detected by PNA-FISH and considering that cultivable cells are in the spiral shape, while coccoid shaped cells are VBNC and therefore likely to be non-cultivable, it would be expected to have more cultivable *H. pylori* at 20ºC, demonstrating that in heterotrophic biofilms the behaviour of this pathogen might be completely different than in pure culture. Additionally, the PNA probe used in this work targets sites on the 16S rRNA molecule and it is known that the RNA content of a cell can be indicative of viability [6], which suggests that the cells detected were still viable. It has been shown above that the concentration of total *H. pylori* included in the biofilm formed in this work is either higher or very similar to the concentration found when pure culture biofilms were formed [5]. In addition, the detection of *H. pylori* embedded in biofilms suggests a close-association with other bacteria present in the biofilm. These two factors, together with the persistence of a bright PNA-FISH signal, indicative of a high rRNA content, suggest that the heterotrophic bacteria present in the biofilm formed in this study are not influencing negatively *H. pylori* but only inducing it's transformation into the more robust coccoid morphology [2].

The mode of transmission of *H. pylori* is not well established, and although there is considerable evidence that water can be a strong candidate, several authors are sceptical in accepting this route of transmission. This work provides new evidence about the survival of *H. pylori* in drinking water biofilms, showing that this pathogen, although being fastidious and losing cultivability easily and rapidly, can still maintain viability in the environmentally robust coccoid VBNC state for long periods of time. The fact that this work, contrary to the pure culture studies reported in the literature, has been done using natural, heterotrophic microbial consortia, shows the capacity of *H. pylori* to adapt to stress situations by "taking advantage" of the presence of other microorganisms.

5.4 Conclusions

This study is in agreement with the data obtained in the aquatic environment, where *H. pylori* is detected by molecular techniques but not by plating methods [9]. In fact, it has been shown here that even in artificially-inoculated systems, *H. pylori* recovery and growth on agar culture plates remains elusive despite the abundant presence of the bacterium in biofilms as assessed by PNA-FISH. On the other hand, previous experiments have shown that water-exposed *H. pylori* NCTC 11637 remains cultivable in pure-culture biofilms for at least 24 hours [3].

The high numbers of *H. pylori* present in biofilms and the maintenance of high levels of rRNA within the cells for at least 31 days strongly suggests that, far from being deleterious, interactions are indeed protecting the pathogen by providing a stable, possibly microaerophilic environment for their cells to subsist. This indicates that *H. pylori* might be found in biofilms in a VBNC state confirming that standard cultivation methods are not the best approach to assess the safety of drinking water in respect to this pathogen, and that while improved recovery methods are not available, it would be important to utilise PNA-FISH as a monitoring method. This work shows that even when cultivable *H. pylori* is not detected by standard methods this pathogen will persist in biofilms under most conditions found in aquatic environments, suggesting that water biofilms might have a role on *H. pylori* transmission.

5.5 Acknowledgements

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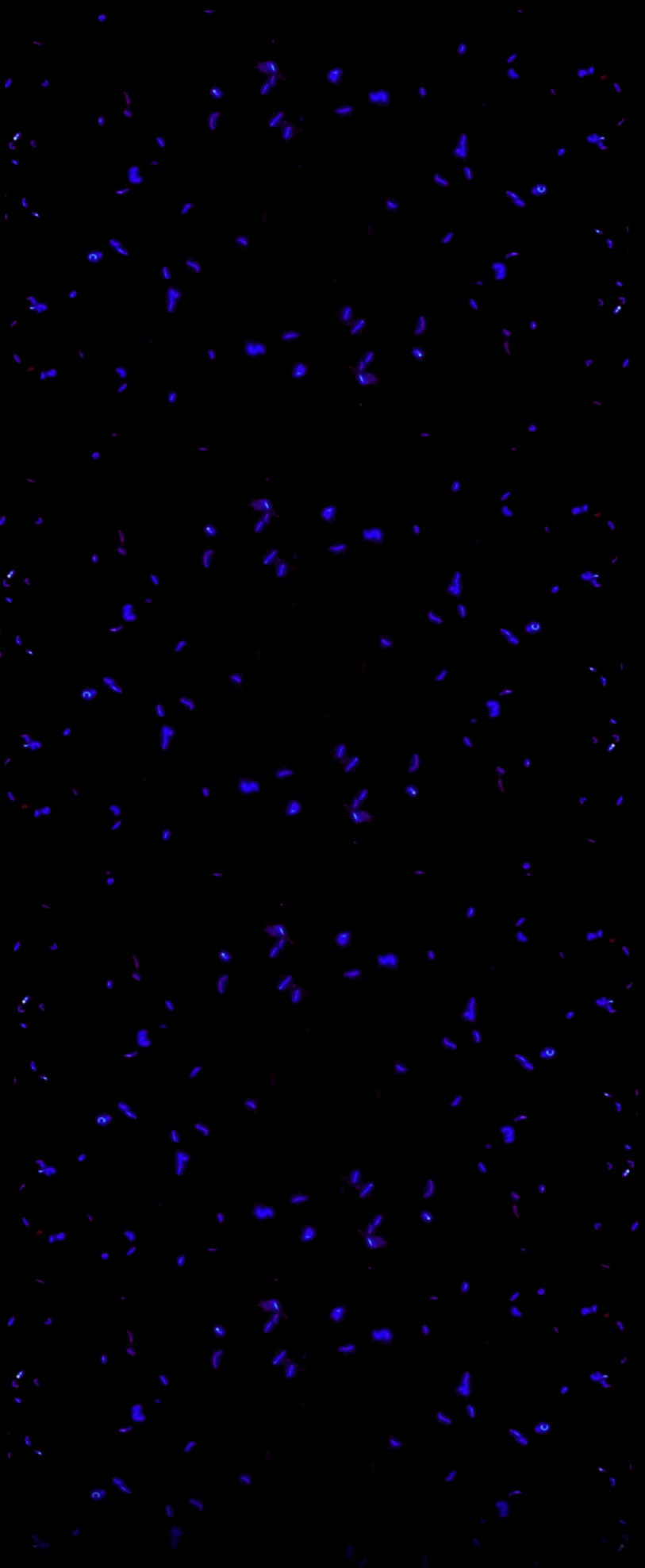

Resistance of *Helicobacter pylori* to chlorine in drinking water biofilms

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Transmission routes of *Helicobacter pylori* between human individuals are not well established but aquatic microenvironments are suspected of playing a role. In this work, the effect of chlorine on *H. pylori* cells embedded in potable water biofilms and when in a pure culture suspension was studied. For the heterotrophic experiments, a two-stage chemostat system was first inoculated with a bacterial consortium obtained from tap water and, after biofilm formation reached a steady-state, the system was challenged with the pathogen. Total *H. pylori* cells were detected using a specific 16S rRNA peptide nucleic acid (PNA) probe and cultivable cells were quantified by plating onto selective HP medium. In experiments with suspended cells, viability was assessed using the SYTO 9/Propidium Iodide fluorochrome uptake assay and cultivability assessed by standard culture techniques. Although cultivable *H. pylori* could not be recovered from biofilms, results using the PNA probe demonstrated that *H. pylori* will persist in chlorinated biofilms for at least 26 days even at the highest concentrations of chlorine (1.2 mg l^{-1}), probably in a viable but noncultivable state. In addition, total *H. pylori* cell numbers appear not to be affected by chlorine concentration when embedded in biofilms, which confirms the extended resistance of this pathogen for chlorine and raises obvious concerns for public health. Experiments with suspended cells showed that *H. pylori* retains cultivability for at least 30 min at 0.2 mg l^{-1}. The lack of cultivability in biofilms was therefore mainly attributed to the overgrowth and interaction of other bacteria in the selective HP medium, which points to the need of further improving selective plating methods for the detection of this pathogen.

6.1 Introduction

Helicobacter pylori is a Gram-negative microorganism that colonizes the human stomach and can cause gastric ulcers that under certain circumstances might degenerate into gastric carcinoma [9]. The route of transmission for this pathogen is not well-known, and even though cultivable *H. pylori* has never been isolated from drinking water distribution systems (DWDS), molecular techniques such as PCR have detected the presence of *H. pylori* DNA in potable water [10, 28, 32], indicating that this environment could act as an environmental reservoir for this bacterium. In fact, epidemiological studies confirm that, particularly in developing countries, untreated water is a risk factor to increase the incidence of this pathogen in the human population (reviewed in [5, 16, 18]).

Chlorine is the most commonly used disinfectant worldwide to ensure a safe distribution of water to the consumer [33]. However, safety of drinking water is usually assessed by the presence of the indicator of faecal pollution, *Escherichia coli*, and coliform bacteria, microorganisms that have been proven to be less resistant to chlorine than several pathogenic bacteria such as *Legionella* spp. [26] and *H. pylori* [8, 20]. Besides showing the extended resistance of *H. pylori* to chlorine, Johnson *et al.* [20] and Baker *et al.* [8] have also shown that *H. pylori* is inactivated by 0.12 and 0.299 mg chlorine $(l\ min)^{-1}$, respectively. However their conclusions were based on the lack of recovery using standard plating methods that fail to consider cells that have entered a viable but non cultivable (VBNC) state. Recently, Moreno et al. [30] applied molecular techniques to demonstrate that *H. pylori* can survive in low concentrations of chlorine in a VBNC state. Nonetheless, all these studies were performed on pure cultures using suspended cells and up until now there have been no studies reporting the effect of chlorination on *H. pylori* when associated with heterotrophic biofilms. It is well-known that microorganisms in biofilms are more resistant to the biocide effect of antibiotics and chlorine due to the difficulty of these molecules to diffuse through the biofilm matrix [14].

Recovery of pathogens by standard cultivation techniques is known to have limitations. On the other hand, fluorescence *in situ* hybridization (FISH) using peptide nucleic acid (PNA) probes has been widely studied in recent years as a promising technique to detect several pathogens in DWDS [7, 21, 27, 35].

The aim of this work was to study the effect of low concentrations of chlorine on *H. pylori* cells, when associated with heterotrophic biofilms and when suspended in pure culture, to assess the ability of biofilms to act as a protective niche for this pathogen.

6.2 Material and Methods

6.2.1 Culture maintenance

H. pylori NCTC 11637 was maintained in vials frozen at -80ºC and recovered by plating onto Columbia Agar (Oxoid, UK) supplemented with 5% (v/v) defibrinated horse blood (CBA) (Oxoid, UK) and incubated for 48 hours at 37ºC in a variable atmosphere workstation (MACS VA500, Don Whitley, UK) set to a microaerobic atmosphere of 10 % CO_2, 7 % H_2 and 3 % O_2. *H. pylori* was subcultured for 48 hours and then inoculated either into the second-stage chemostats (see below) or used in the pure culture cells assessment.

6.2.2 Chlorine preparation and measurements

Chlorine tablets (H-8801, Guest Medical, UK) were added to filtered distilled water to obtain a 5 g l^{-1} stock solution. The measurement of chlorine was performed by the N,N-dimethyl-p-phenilenediamine (DPD) colorimetric method, as described in the Standard Methods for the Examination of Water and Wastewater [2] with the exception of the absorbance wavelength reading which was adjusted to 492 nm [29].

6.2.3 Experiments in heterotrophic biofilms

6.2.3.1 Biofilm formation

The formation of biofilms was carried out using a two-stage chemostat model system. The first stage consisted of a 1-litre vessel (seed vessel) and the second stage consisted of three 1-litre vessels running in parallel, but connected in series with the seed vessel [22]. All chemostats were autoclaved and filled with filter-sterilized tap water (1-litre). The seed vessel was then inoculated with a microbial consortium that was obtained from tap water by filtration through a 0.2 μm pore size Nylon filter (Pall Gelman, UK), maintained in batch mode for two days to promote microbial growth and then changed into a continuous mode, being fed with filter-sterilized and dechlorinated tap water at a flow rate of 50 ml h^{-1}. This chemostat was operated at room temperature and stirred at 300 rpm to ensure that the oxygen and nutrient concentrations were homogeneous. The outflow was divided in three and used to feed the second stage chemostats – the biofilm-growing vessels. Each biofilm-growing vessel was

also fed with fresh media (filter-sterilized tap water) at a flow rate that maintained the dilution rate of 0.2 h^{-1} to promote the typical environmental conditions for biofilm growth. All vessels were stirred at 300 rpm and the temperature was controlled at 15ºC by a proportional integral derivative unit system (Brighton Systems, UK). To one of the biofilm-growing vessels no chlorine was added, serving as a control, and to the other two vessels chlorine was continuously supplemented, with the concentration being maintained at 0.2 mg l^{-1} and 1.2 mg l^{-1}. After 10 days, conditions in the biofilm-growing vessels were stable and these were inoculated with *H. pylori* NCTC 11637 at a final concentration of approximately 3.18 x 10^5 CFU ml^{-1} followed by the immersion of sterile unplasticized polyvinylchloride (uPVC) coupons (day 0). The coupons were removed after 1, 2, 4, 8, 16 and 26 days, gently rinsed to remove planktonic cells attached to the surface of the biofilm, and scraped to quantify sessile cells.

6.2.3.2 Coupon preparation

One cm^2 uPVC coupons were used as a substratum for biofilm growth. Coupons were immersed in water and detergent for 5 minutes, washed with a bottle brusher, rinsed twice in distilled water and air-dried. Subsequently, they were washed in 70% (v/v) ethanol to remove any organic compounds, attached to the end of a titanium wire and autoclaved [22].

6.2.3.3 Quantification of planktonic cells

Water samples were taken after 0, 1, 2, 4, 8, 16 and 26 days from the seed and biofilm-growing vessels and were analyzed for total cells, heterotrophic cells and cultivable *H. pylori*. Total cells were quantified using the SYTO 9 staining method (Molecular Probes, Invitrogen, UK). In short, 1 ml of an appropriate dilution was mixed with 0.5 μl of SYTO 9, incubated in the dark for 15 minutes, filtered through a 0.2 μm pore size polycarbonate black Nucleopore® membrane (Whatman, UK) and allowed to air-dry. Then a drop of non-fluorescence immersion oil (Fluka, UK) and a coverslip were added before observation under a Nikon Eclipse E800 episcopic differential interference contrast/epifluorescence (EDIC/EF) microscope (Best Scientific, UK) [23]. As the cells were homogenously distributed, fields of view were chosen at random and the number of cells counted on each membrane. Heterotrophs were quantified by heterotrophic plate count (HPC) by plating onto R2A (Oxoid, UK) and incubated at 22ºC for 7 days. Cultivable *H. pylori* was quantified by plating onto HP medium agar plates as described in Chapter 5 and incubated at 37ºC in a microaerophilic atmosphere

for 7 days. The colonies obtained on HP agar plates were tested with the urease test (Oxoid, UK) and by the use of a specific PNA probe to confirm the identity of *H. pylori* (see below).

6.2.3.4 Quantification of sessile cells

Coupons were immersed in 2 ml of filter-sterilized tap water containing autoclaved 2 mm diameter glass beads (Merck, UK) and vortexed for 1 min to remove all the biofilm from the coupons surface and homogenize the suspension. Total cells, HPC and cultivable *H. pylori* were quantified using the methods described above. In addition, total *H. pylori* were quantified using a specific PNA probe with the following sequence 5'- GAGACTAAGCCCTCC -3' (Eurogentec, Belgium) in a fluorescence *in situ* hybridization assay (PNA-FISH) [19]. PNA-FISH was carried out by taking 1 ml of an appropriate dilution and filtered through a 0.2 μm anodisc membrane (Whatman, UK). This was left to air dry. Then the membrane was covered with 4% (w/v) paraformaldehyde followed by 50% (v/v) ethanol for 10 minutes each to fix the cells and air dried. The hybridization, washing and microscopy observation method was performed as described by Guimarães et al. [19].

6.2.3.5 Chlorine measurements and inactivation

Two of the biofilm-growing vessels were also continuously fed with chlorine, provided by 2 stock solutions prepared every two days from a 5 g l^{-1} stock solution (Guest Medical, UK). Chlorine concentration was controlled on a daily basis by measuring free chlorine on the water vessels by the N,N-dimethyl-p-phenilenediamine (DPD) colorimetric method, as described before. To quantify planktonic and sessile cells from these two vessels, it was necessary to neutralize the chlorine. As such, sodium thiosulfate (Sigma, UK) at a final concentration of 5 mg l^{-1} was added to all the samples.

6.2.4 Experiments with *H. pylori* in pure culture

6.2.4.1 Chlorine disinfection tests

After considering the chlorine demand due to organic matter, an appropriate amount of the stock solution was added to a suspension containing approximately 10^6 *H. pylori* CFU ml^{-1}, in order to obtain a free chlorine final concentration of 0.2; 0.7 and 1.2 mg l^{-1}. A control assay where no chlorine was added was also performed. Experiments were carried out in amber flasks (to avoid chlorine

degradation by light) at room temperature and stirred at 620 rpm. Samples were taken at 0, 10, 20 and 30 minutes and cells quantified as explained below. At times 0 and 30 minutes the concentration of free chlorine was measured by the DPD method as described before. The chlorine reaction was inactivated by the addition of sodium thiosulfate (Sigma, UK) applied at a final concentration 5 mg l^{-1}. For each chlorine concentration the experiment was repeated at least three times.

6.2.4.2 Assessment of cultivable cells

A 40 μl aliquot of the previous samples was diluted (to obtain between 15 and 150 CFU per agar plate) and spread onto CBA agar plates (in triplicate for each experiment) and incubated at 37ºC, in the same atmosphere as described before, for 7-14 days. After this time the number of colonies was counted to determine the number of cultivable cells remaining in the chlorinated solution.

6.2.4.3 Assessment of membrane integrity

To assess membrane integrity the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit (Molecular Probes, UK) was used. A 50 μl aliquot of the samples was diluted in 0.95 ml of dechlorinated filtered tap water and stained with SYTO 9/PI. A 3 μl volume of an equal proportion of SYTO 9 and PI mixture was added to the sample and incubated in the dark, at room temperature for 15 minutes followed by filtration through a polycarbonate black Nucleopore® membranes (0.2 μm pore size) (Whatman, UK). Subsequently, the membranes were air dried, mounted onto glass slides with non-fluorescence immersion oil and a cover slip. The slides were examined using an episcopic differential interference contrast/epifluorescence microscope (EDIC/EF) (Best Scientific, UK) [23].

6.2.4.4 DNA electrophoresis

H. pylori NCTC 11637, grown under the same conditions as previously described, was suspended in 100 ml of dechlorinated and filtered tap water to give a final concentration of approximately 10^6 CFU ml^{-1}. This cell suspension was exposed to the same range of chlorine concentrations for 30 minutes. Following this, cells were concentrated by centrifugation at 4000 rpm for 10 minutes and the DNA extracted and purified using a DNA extract kit (Sigma, Spain). The DNA obtained was run in a horizontal electrophoresis system for 2 hours at 100 V using 1% (w/v) agarose gel (Bio-Rad, Portugal)

containing ethidium bromide (50 $\mu l\ l^{-1}$) of a 10 mg ml^{-1} stock (Bio-Rad, Portugal). Finally, the gel was visualized by UV light.

6.2.5 Statistical analysis

Results obtained cultivable cells, membrane integrity, RNA injury, total and PNA cell counts were transformed using a $\log_{10}$ scale. The mean for each was calculated based on at least three experiments, and the homogeneity of variance across these parameters was checked by the Levene test for equality of variances using a statistical package (SPSS Inc., Chicago IL, USA). Differences between the parameters measured were subsequently compared by a one-way ANOVA followed by a Bonferroni post hoc test. Differences were considered relevant if $P<0.05$.

6.3 Results

6.3.1 Planktonic cells in the two-stage chemostat

Prior to the immersion of coupons the two-stage chemostat was continuously operated for ten days to stabilize the microbial consortia in all the vessels. Afterwards and during the entire experiment the number of total cells in the seed vessel remained constant with time but the number of heterotrophic bacteria (HPC) decreased slightly ($P<0.05$), with an average 2.39×10^6 cells ml^{-1} and 7.10×10^5 CFU ml^{-1}, respectively. On Table 6.1 the mean of values obtained for total cells and HPC in the planktonic phase for the three second-stage chemostats are presented. The numbers of total cells were significantly lower ($P=0.001$), on average 40% lower in the chemostats where chlorine was added than in the control vessel, where no chlorine was added (2.16×10^6 cells ml^{-1}). However, no statistically significant differences were observed between the two concentrations of chlorine used (1.29×10^6 cells ml^{-1} and 1.22×10^6 cells ml^{-1}, in the vessels where the concentration of chlorine was 0.2 mg l^{-1} and 1.2 mg l^{-1}, respectively). Concerning the HPC numbers, the values obtained decreased smoothly during the experiment (in general $P<0.05$), and were higher in the control vessel (9.88×10^5 CFU ml^{-1}) and lower in the vessel with the highest concentration of chlorine (3.47×10^4 CFU ml^{-1}). The HPC were statistically different when the three vessels were compared ($P=0.001$). The absence of autochthonous *H. pylori* in the seed vessel required the inoculation of the biofilm growing vessels with a collection strain as described above. However it was never possible to recover cultivable *H. pylori* from any of the chemostats inoculated. Nevertheless, overgrowth of other microorganisms was observed on the HP agar medium.

Table 6.1 Average numbers of total cell and HPC in the planktonic phase for all three conditions tested.

Cl_2 concentration (mg l^{-1})	**Total cells x 10^{-6} (cell ml^{-1})**	**HPC x 10^{-5} (CFU ml^{-1})**
0.0	2.16	9.88
0.2	1.29	1.37
1.2	1.22	0.347

6.3.2 Population of biofilms

Figure 6.1a shows that most of the microorganisms adhered to the surface during the first day, and that afterwards the numbers did not significantly change (in general, P>0.05). It was also possible to observe that the total numbers of cells that constitute the biofilm in the absence and presence of chlorine are different (P<0.05) but similar for the two different concentrations of chlorine used. The numbers of total *H. pylori* detected by the specific PNA probe did not change significantly with the 3 conditions tested (P>0.05) (Figure 6.1b). It was in terms of cultivable cells that the differences between the three conditions tested were observed. As expected, HPC decreased significantly when the concentration of chlorine increased (Figure 6.1c). The HPC numbers were lower for the highest concentration of chlorine (3.22 x 10^2 CFU cm^{-2}) comparing to the other concentration of chlorine (9.94 x 10^2 CFU cm^{-2}), and almost 3-log lower than in the control vessel (1.54 x 10^5 CFU cm^{-2}). Once again, no cultivable *H. pylori* were detected although overgrowth of other microorganisms on the HP media was observed.

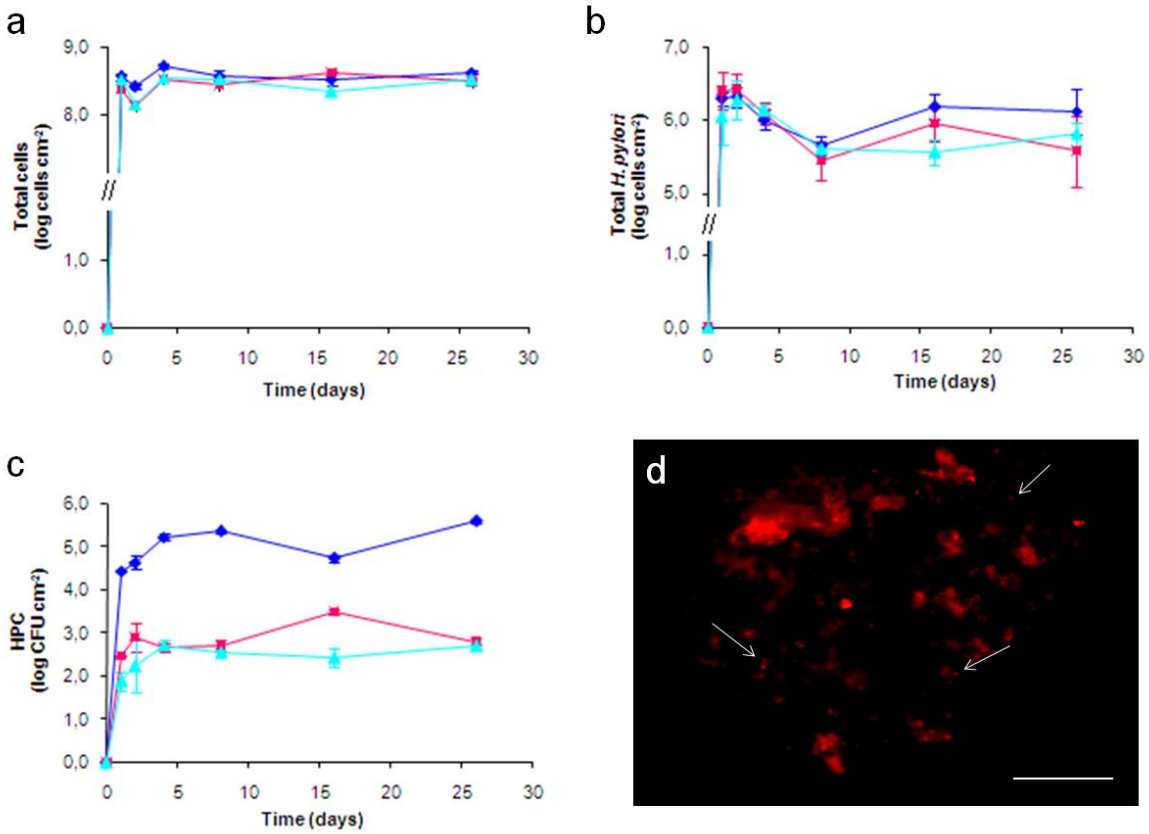

Figura 6.1. Variation in the total cell number (a), total numbers of *H. pylori* (b) and HPC (c) in biofilms formed when no chlorine is added (◆), when chlorine is continuously added to a final concentration of 0.2 (■) and 1.2 (▲) mg l^{-1}. Epifluorescence microphotograph of a biofilm hybridized with the *H. pylori* specific PNA probe, using the TRITC filter. Bar represents 20 μm (d).

6.3.3 Effect of chlorine on pure *H. pylori* suspensions

In order to understand the contribution of the heterotrophic consortium to protect *H. pylori* cells from chlorine, an additional set of experiments using pure *H. pylori* cultures in suspension was performed. Unlike the experiments on biofilms, chlorine was only added at the beginning of the experiment and as such it was completely consumed after 30 minutes of contact time when the concentration of free chlorine used was 0.2 and 0.7 mg l^{-1}, whereas for the highest concentration of chlorine (1.2 mg l^{-1}) there was still some chlorine remaining in solution (Table 6.2). The values of the concentration of chlorine presented (0.2; 0.7 and 1.2 mg l^{-1}) already consider the chlorine demand due the initial combination with organic matter.

Table 6.2 Chlorine concentration demand immediately after and 30 minutes after the chlorine addition to the *H. pylori* suspension.

Cl_2 concentration (mg l^{-1})	**Cl_2 measurement in the cell suspension (mg l^{-1})**	
	0 min	**30 min**
0.2	0.373	0.076
0.7	0.598	0.044
1.2	1.011	0.115

In the control experiment, where no chlorine was added to the cell suspension, the number of total (sum of viable and non-viable) and cultivable cells appear to remain constant with time, meaning that at 20ºC there was no effect of water exposure during the 30 minutes of experiment (Figure 6.2a). For all concentrations of chlorine added, there was no decrease in the number of viable cells though some of the stained cells fluoresced from bright green to yellow and orange. The morphology of cells remained constant during the entire length of the experiment for all conditions tested. The addition of chlorine led to some loss of cultivability. It was observed that cells lost some cultivability when chlorine was added but never completely lost the cultivability even for the highest concentration of chlorine. Moreover, the difference between the number of cultivable *H. pylori* following 0.2 and 0.7 mg l^{-1} chlorine stress was not statistically significant ($P>0.05$). A surprising result was the disappearance of the non-viable (cells that fluoresce red) bacteria for the two highest concentrations of chlorine used (Figure 6.3b and c). For 0.7 mg l^{-1} the number of non-viable cells started decreasing after 10 minutes

of exposure time and completely disappeared in 10 minutes. For the highest concentration used the non-viable cells completely disappeared during the first 10 minutes of chlorine exposure.

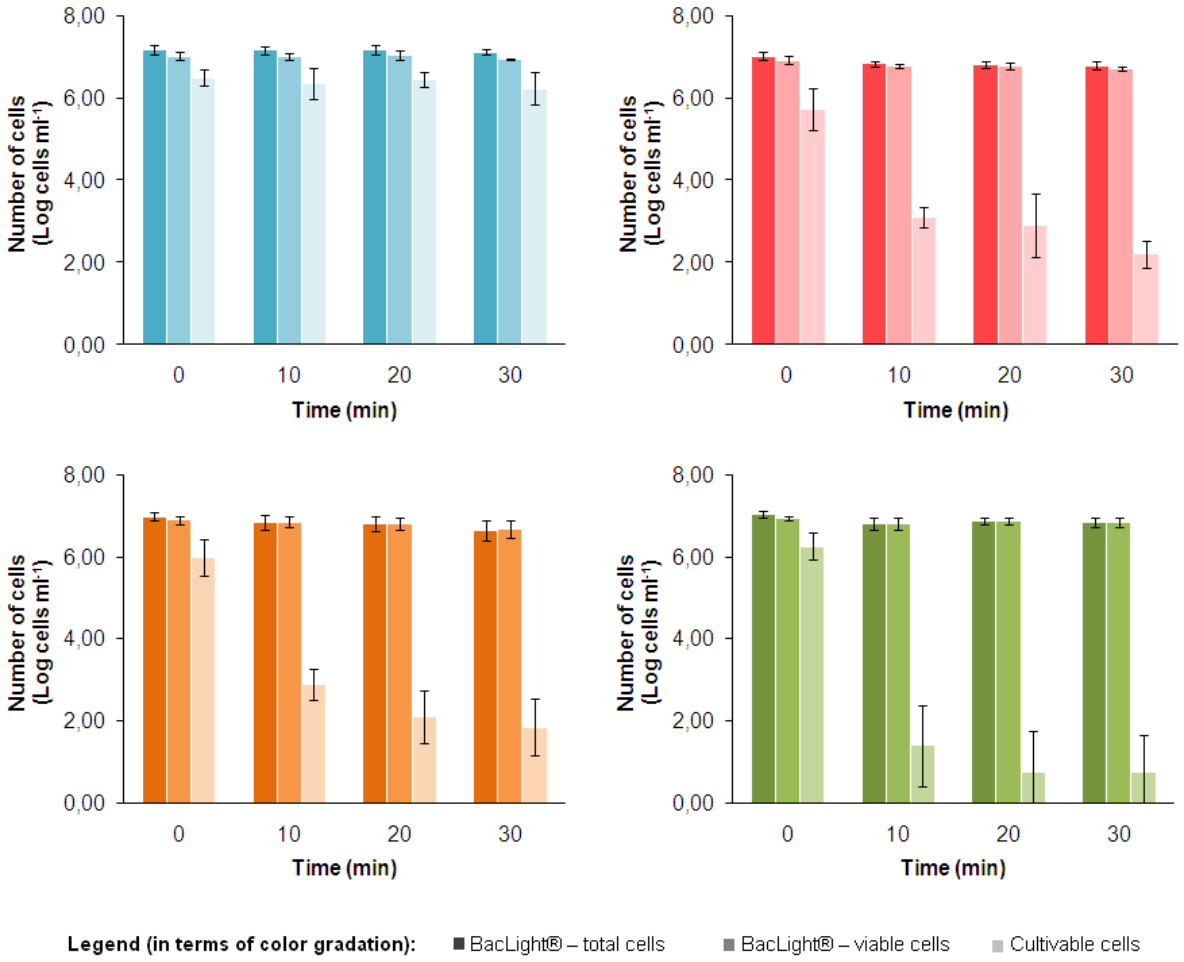

Figura 6.2. Variation in the number of total, viable and cultivable cells, after exposure to 0.0 (a), 0.2 (b), 0.7 (c) and 1.2 (d) mg l^{-1}. Error bars represent standard deviation of at least three experiments.

DNA electrophoresis (Figure 6.3d) showed that after 30 min there were no cuts in the DNA, as only one band appeared in the gel for all the conditions tested. However the bands became faint at chlorine concentrations of 0.7 and 1.2 mg l^{-1}.

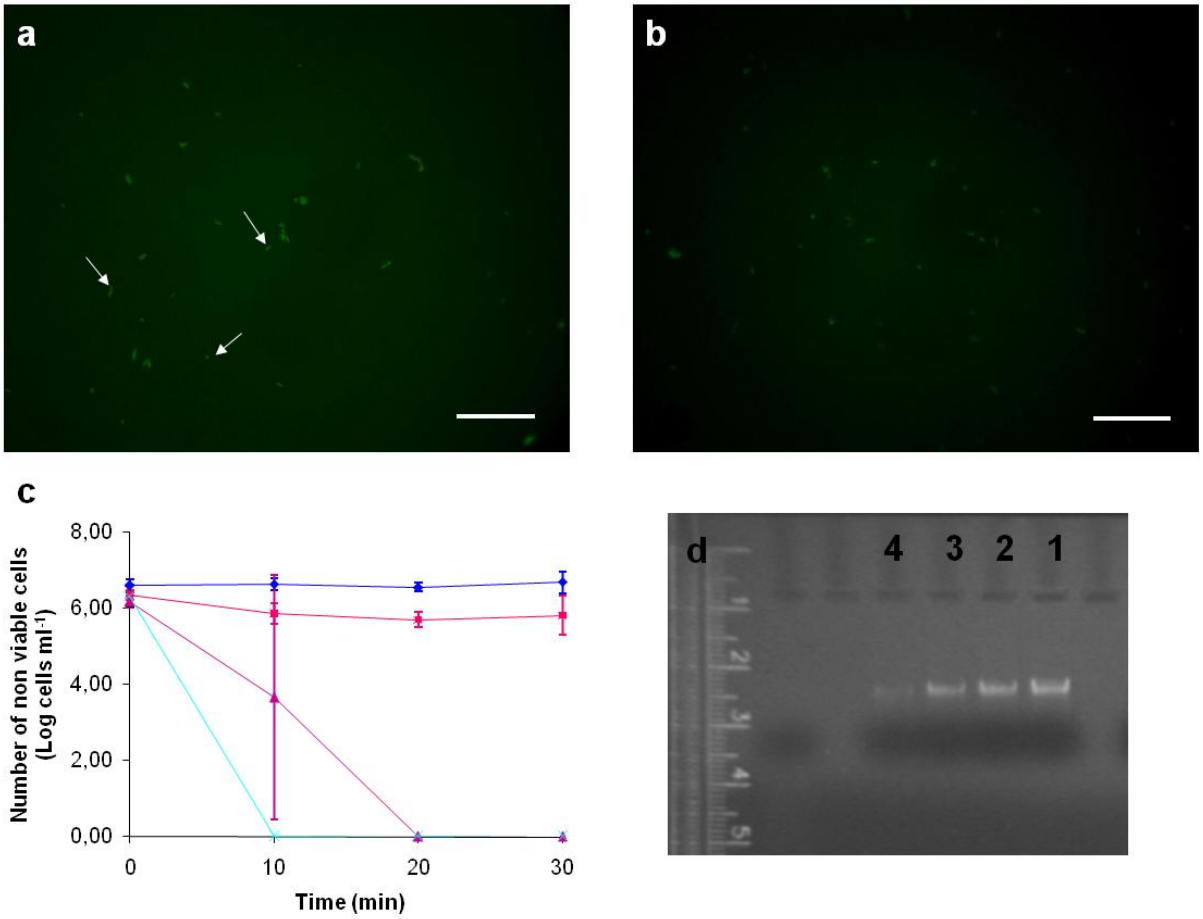

Figura 6.3. Epifluorescence microphotograph showing *H. pylori* cells treated with 1.2 mg l^{-1} of free chlorine and stained with LIVE/DEAD® *Bac*Light™ bacterial viability kit, at time 0 (a) and 30 minutes (b); Bars represent 20 μm and arrows indicate PI positive cells. Variation in the numbers of non viable cells after exposure to 0.0 (◆), 0.2 (■), 0.7 (▲) and 1.2 (x) mg l^{-1} of free chlorine. Error bars represent standard deviation of at least three experiments(c). Chromosomal DNA bands isolated from *H. pylori* cells after exposure to 0.0 (1); 0.2 (2); 0.7 (3) and 1.2 (4) mg l^{-1} of free chlorine (d).

6.4 Discussion

6.4.1 Planktonic cells in the two-stage chemostat

To form the heterotrophic biofilm a microbial consortium obtained by filtering tap water was used and growth was promoted by using a low flow rate in the seed vessel. The constant numbers of total cells and HPC for over 10 days revealed that the seed vessel was stable. *H. pylori* was not one of the microorganisms present in this consortium, and as such the biofilm growing vessels were spiked with a collection strain to study the influence of chlorine on the incorporation of this pathogen into drinking water biofilms. It is known that at 15ºC this bacterium can maintain cultivability in suspension for more than 70 hours [1, 4]. However, 30 minutes after inoculation, the water from all biofilm-growing vessels was analyzed and no *H. pylori* was recovered on HP media but the growth of other microorganisms was observed. As such, this strongly indicates that the recovery failed due to the overgrowth of heterotrophic microorganisms and not to the immediate loss of cultivability by *H. pylori*. The medium developed by Degnan et al. [15] needs improvement to be used in samples collected from environment, as it has also been shown by Fernández and colleagues [17].

Results show that the total number of cells obtained in the second stage was similar to those obtained in the first stage, despite the former being diluted with fresh medium. These results were observed before the formation of biofilm, indicating that these high numbers were due to planktonic growth and not to interactions with the biofilm. In fact, different concentrations of chlorine seem to have little effect on the total number of planktonic microorganisms as the numbers obtained were similar, for the two concentrations used and approximately half of the value obtained for the control vessel. The same does not happen in terms of cultivability as cells lost cultivability with increasing concentrations of chlorine. The fact that there are still cultivable cells suggests that there are microorganisms in the inoculum that are resistant to chlorine and able to be recovered on artificial medium, which is not surprising as the inoculum was obtained from chlorinated municipal water which means that bacteria were chlorine adapted.

6.4.2 Sessile cells in the second stage of the chemostat system

The quantification of total cells present in the biofilm showed that different concentrations of chlorine had no effect on biofilm formation as the numbers were similar for the two concentrations tested and during the 26 days of experiment, which is not an unexpected result as it has been demonstrated previously that biofilms are able to grow even in the presence of chlorine [24, 31]. The differences observed here were in terms of cultivable cells, as the addition of chlorine promoted a loss of cultivability of almost 3-log. These results can be supported by two studies conducted by Codony et al. [11, 12], where it was shown that under chlorinated conditions the total number of cells was similar to the control study but cells were less cultivable.

6.4.3 Inclusion of *H. pylori* in heterotrophic biofilms

The quantification of total *H. pylori* showed that in the first few days there was no significant difference between the different conditions tested, suggesting that, as the inoculum added to the chemostats was the same, the inclusion of *H. pylori* in the heterotrophic biofilm was not influenced by the presence of chlorine. During the first week there was a decrease in the number of total *H. pylori* that can be explained by the fact that the pathogen has just been inoculated in the beginning of the experiment (prior the immersion of coupons) and planktonic *H. pylori* cells were theoretically expected to be washed out after approximately five hours. As such, after this time the cells that detach from the biofilm can not be replaced and the detachment of biofilm that sloughs off *H. pylori* cells leads to a decrease in the total numbers of this pathogen. After one week of biofilm formation the numbers of *H. pylori* are constant for all the conditions tested although slightly lower in the biofilms formed under chlorinated conditions. This might happen because the biofilm formed in the control chemostat were slimier than the biofilms formed under chlorinated conditions. This slimy aspect indicates the presence of exopolymeric substances (EPS) that embedded the biofilm, protecting it from external stress conditions such as exposure to biocides. On the other hand, when the biofilm is thicker the detachment of biofilm portions occurs preferentially in the external layer while internal layers remain intact. The attachment of *H. pylori* occurred in the first 5 hours of biofilm formation, as it has been explained before, and incorporates within in the inside layers. In thicker biofilms these layers remain intact so the numbers of total *H. pylori* are constant with time. Conversely, when chlorine is added biofilms are thinner so the detachment may also occur in the layers where *H. pylori* is adhered. On the other hand, chlorine promotes biofilm detachment which can also contribute to a decrease in *H. pylori* numbers.

Microscopy observation of *H. pylori* labeled to the 16S rRNA probe showed that the morphology of the cells was mostly coccoid, both in the absence and presence of chlorine. This might be an important observation as coccoid cells are known to retain their viability for longer and still able to cause infections [3, 34]. Previous results have demonstrated that coccoid is the preferred shape at 15ºC while at 20ºC the cells are normally spiral shaped (Chapter 5). This might indicate that at 15ºC cells are more resistant to chlorination, which is the most commonly used disinfectant in DSWS.

6.4.4 Effect of chlorine on pure *H. pylori* suspensions

In the experiments described above two factors may have influenced the response of *H. pylori* to chlorination: the presence of other microorganisms that might influence the behaviour of the pathogen and the inclusion in biofilms, where *H. pylori* is more protected from the biocide effect of chlorine. To try to understand the effect of this disinfectant on *H. pylori* cells, studies on suspended cells using a pure culture were carried out. The studies were performed with suspended cells as other authors have already demonstrated the inability of *H. pylori* to form homogeneous monospecies biofilms under most conditions when suspended in water [3, 6, 13]. It was observed that *H. pylori* lost cultivability in the presence of chlorine ($P<0.05$) for all the concentrations used compared to the control experiment but,

even at the highest concentration, it was always possible to recover cultivable cells. This could had been due to the combination of organic matter that was introduced in the suspension with the cells, as suggested by Johnson and colleagues [20], when they found that *H. pylori* was more resistant than *E. coli*. However chlorine was measured at the end of the experiments and it was observed that for the experiment at 1.2 mg l^{-1} there was still chlorine in the suspension, which means that there was enough chlorine to react with the cells. On the other hand, the results presented in this study are opposite to those obtained by other authors [8, 20, 30] where *H. pylori* had completely lost cultivability even at lower concentrations than 1.2 mg l^{-1}. This suggests that other parameters such as temperature, light and water characteristics are important in chlorination efficiency [4].

The viability analysis showed that even for 1.2 mg l^{-1} the number of viable cells remained constant. An interesting result was the fact that the non-viable cells disappeared when the concentration of chlorine was 0.7 and 1.2 mg l^{-1}. It was also observed by DNA electrophoresis that for these two concentrations the genomic bands were fainter meaning that the concentration of DNA decreased. These two results combined suggest that weaker *H. pylori* cells are more susceptible to chlorine than stronger cells, e.g. there are *H. pylori* altruistic cells which absorb the chlorine to protect the others.

The results obtained in pure culture can bring some highlights for what happened in the experiments with biofilms. The failure to recover cultivable *H. pylori* was likely to be due to the fact that the isolation medium was not completely selective for *H. pylori*, allowing other microorganisms to grow. As a fastidious microorganism that grows very slowly, the presence of other species may easily overgrow *H. pylori*, being impossible to obtain cultivable data that could give important information. On the other hand biofilms seem to promote altruism in cells [25], meaning that in biofilms *H. pylori* viable cells might be extra protected by weakest *H. pylori* cells but also by other microorganisms present in the consortium. The disappearance of non viable cells and loss of brightness of the DNA bands suggests that the decrease in the numbers of total *H. pylori* in biofilms were not only due to the detachment of the cells but also to the direct action of chlorine on altruistic cells. The detection of rRNA after 26 days of inoculation indicates the presence of viable cells. It has been shown that cells can maintain bright rRNA fluorescence after some hours of death but it would be impossible for 26 days. These two results indicate that most dead cells disappeared in the first days of chlorine exposure and the cells that were detected afterwards by PNA-FISH can be considered viable, although probably in VBNC state, corroborated by the fact that most of the cells are coccoid shaped.

The fact that *H. pylori* in pure culture has never completely lost its cultivability shows the resistance of this strain to chlorine, and the extra protection of biofilms which can lead to the presence of viable *H. pylori* in DSDW, even if the cells are non cultivable. This work strongly supports the view that chlorine in water might inhibit the activity of *H. pylori*, but fails to eliminate the pathogen from DWDS biofilms, a safe haven where the pathogen might survive in a VBNC state.

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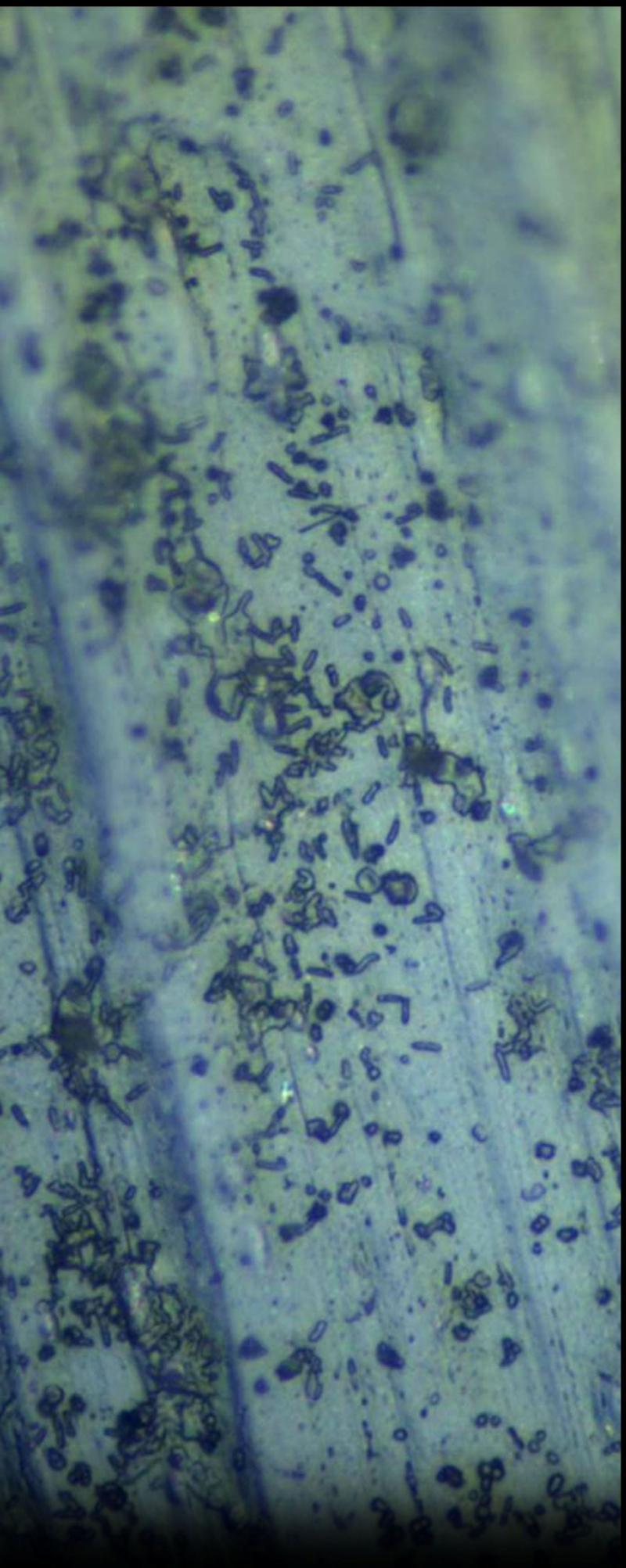

Interaction of *Legionella pneumophila* and *Helicobacter pylori* with bacterial species isolated from drinking water biofilms

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Drinking water biofilms provide an ecological niche for several pathogens, such as *Legionella pneumophila* and *Helicobacter pylori*, to survive stress conditions. It is well established that *L. pneumophila* is a waterborne pathogen; by contrast, the mode of of *H. pylori* transmission remains unknown, but water seems to play an important role. This work aims to study the influence of several microorganisms isolated from drinking water biofilms on the survival and integration of both pathogens into biofilms. For that, dual-species biofilms were grown with *L. pneumophila* or *H. pylori* and each one of the isolated strains. A negative control was also performed that consisted of a biofilm formed only by *L. pneumophila* or *H. pylori* in pure culture. In all experiments sessile cells were quantified in terms of total cells by SYTO 9 staining, total *L. pneumophila* or *H. pylori cells* using 16S rRNA-specific peptide nucleic acid probes and cultivable cells by standard culture techniques. Most heterotrophic plate count (HPC) bacteria appear to have an antagonistic effect on *L. pneumophila* cultivability but not viability, leading to the formation of viable but noncultivable (VBNC) cells, whereas *Mycobacterium chelonae* increased the cultivability of this pathogen. *M. chelonae* and *Sphingomonas* spp. help *H. pylori* maintain cultivability for longer. It therefore appears that *M. chelonae* has an important role in the survival of both pathogens in drinking water. This work also suggests that the presence of some microorganisms can decrease the cultivability of *L. pneumophila* but not the viability, which indicates that the presence of autochthonous microorganisms can lead to misleading results when the safety of water is assessed only by cultivable methods.

7.1 Introduction

Under stressful conditions bacteria can adhere to surfaces forming a complex structure called biofilm. In these structures the microorganisms are less exposed to the external factors responsible for stress, such as temperature, low nutrients, presence of biocides, etc [11, 13, 16, 19, 27]. In natural environments biofilms are constituted by several species of microorganisms that can interact with each other either positively (for instance, the synthesis of a metabolite by one species that can be used in the metabolism of another) or negatively (such as nutrient competition) [12, 37, 49]. One type of biofilm that has been widely studied is that formed in drinking water distribution systems (DWDS), because of its role in introduction of pathogens in drinking water and consequent impact in the human health [7, 42].

Legionella pneumophila is a waterborne pathogen that can cause Legionnaires' disease or Pontiac fever [30, 35]. This pathogen is found naturally in fresh water reservoirs and can contaminate drinking water when the disinfection is inefficient, being transmitted to man when contaminated aerosols are inhaled [15, 22, 23, 40]. On the other hand, the mode of transmission of *Helicobacter pylori* remains controversial but drinking water as a route of transmission has been recently proposed. Although no cultivable *H. pylori* has ever been recovered from drinking water systems, molecular techniques such as PCR [9, 29, 34, 46] and peptide nucleic acid (PNA) probes used to target 16S rRNA in fluorescence in situ hybridization (FISH) assays [8, 36], have demonstrated the presence of this pathogen in DWDS. This identification, associated with epidemiological studies that point to different prevalences of *H. pylori* in a population associated with the type of source water, strongly supports water as a route of transmission (reviewed in [3, 17, 20, 26]).

Previous studies have demonstrated that both pathogens can incorporate into heterotrophic drinking water biofilms and remain for at least 32 days (Chapter 3 to 6). In the case of *H. pylori,* although no cultivable cells were ever recovered, the presence of a high intracellular rRNA content indicates that cells might be in a viable but non cultivable (VBNC) state [4]. On the other hand, after incorporation into a multi-species biofilm, it is possible that some of the microorganisms might have contributed for the loss of cultivability [43]. It is also possible that there were other microorganisms present that could have a beneficial effect on *L. pneumophila* or *H. pylori*, as shown by planktonic studies in liquid media [41, 47]. However, for multi-species biofilms it is technically very challenging to determine which sessile microorganisms could have a positive or negative effect on these pathogens, particularly regarding the intimate associations that occur within biofilms. These associations can occur between the same cells (autoaggregation) or between different species (coaggregation) and have been well described for isolates of dental plaque species in complex media and aquatic species in potable water [10, 38].

Therefore, in addition to studying autoaggregation and coaggregation in planktonic culture, some of the microorganisms isolated from these biofilms were used to form dual species biofilms with *L. pneumophila* and *H. pylori* as a way to understand the mechanisms of sessile survival of these two pathogens in drinking water biofilms.

7.2 Material and Methods

7.2.1 Culture maintenance

All strains were maintained in vials frozen at -80ºC and recovered by standard plating procedures onto the appropriate media. *L. pneumophila* NCTC 12821, *Variovorax paradoxus* and *Mycobacterium chelonae* were grown on Buffered Charcoal Yeast Extract (BCYE) agar (Oxoid, UK) for 24 hours at 30ºC. *Acidovorax* sp. and *Sphingomonas* sp. were grown on R2A (Oxoid, UK) for 48 hours at 22ºC. *H. pylori* NCTC 11637 and *Brevundimonas* sp. were grown on Columbia Agar (Oxoid, UK) supplemented with 5% (v/v) defibrinated horse blood (CBA) (Oxoid, UK) and incubated for 48 hours at 37ºC in a microaerophilic atmosphere of 10 % CO_2, 7 % H_2 and 3 % O_2, the remainder being N_2.

7.2.2 Co-aggregation in test tubes

All bacterial strains were suspended in dechlorinated and filtered tap water in a final concentration of approximately 2 x 10^8 cells ml^{-1}. For auto-aggregation, 3 ml of each suspension was transferred into a sterile test tube, whereas for co-aggregation experiments 1.5 ml of either *L. pneumophila* or *H. pylori* suspension was added to 1.5 ml of each one of the species isolated from drinking water biofilms. At times 0, 1, 2, 4, 6, 8, 24 and 48 hours, tubes were vortexed for 10 seconds and observed for co-aggregation according to the scale described by [38].

7.2.3 Biofilm formation

Cells were suspended in 50 ml of dechlorinated and filtered tap water to give a final concentration of approximately 10^7 cells ml^{-1}. As a control , pure-culture biofilms were formed by *L. pneumophila* NCTC12821 and *H. pylori* NCTC11637. In the other experiments, *L. pneumophila* or *H. pylori* were mixed with the other bacteria to form two-species biofilms. All suspensions were homogenized by vortexing and 5 ml were transferred to 6-well microtitre plates containing one unplasticized polyvinylchloride (uPVC) coupon in each well. Plates were incubated in the dark at 22ºC and two coupons of each biofilm type were removed after 1, 2, 4, 8, 16 and 32 days, and gently rinsed to remove loosely attached cells on the surface of the biofilm. One coupon was used for direct observation under a Nikon Eclipse E800 episcopic differential interference contrast/epifluorescence (EDIC/EF) microscope (Best Scientific, UK) [25] using the EDIC channel to directly visualise biofilm and the other one scraped to quantify sessile cells.

7.2.4 Preparation of coupons

uPVC coupons (1 cm^2) were used as a substratum for biofilm growth. Coupons were immersed in water and detergent for 5 min, washed with a bottle brusher, rinsed twice in distilled water and air-

dried. Subsequently, they were washed in 70% (v/v) ethanol to remove any organic compounds and autoclaved at 1 atm and 121ºC [24].

7.2.5 Quantification of sessile cells

Coupons were immersed in 2 ml of filter-sterilized tap water containing autoclaved 2 mm diameter glass beads (Merck, UK) and vortexed for 1 min to remove all the biofilm from the coupons surface and homogenize the suspension. Total cells were quantified using the SYTO 9 staining method (Molecular Probes, Invitrogen, UK). In short, 1 ml of an appropriate dilution was mixed with 0.5 μl of SYTO 9, incubated in the dark for 15 minutes, filtered through a 0.2 μm pore size polycarbonate black Nucleopore® membrane (Whatman, UK) and allowed to air-dry. Then, a drop of non-fluorescent immersion oil (Fluka, UK) and a coverslip were added before observation under the Nikon Eclipse E800 EDIC/EF microscope (Best Scientific) [25]. As the cells were homogenously distributed, 10 fields of view were chosen at random and the number of cells counted on each membrane. Cultivable numbers, obtained for all bacteria, were determined by plating 40 μl of the suspension on the respective agar medium under the appropriate incubation conditions, as described above. BCYE plates were incubated for 2 days and R2A and CBA plates were incubated for 7 days.

L. pneumophila was also quantified using the specific PNA probe PLPNE620 (5'-CTG ACC GTC CCA GGT-3') and *H. pylori* by the use of a PNA probe with the following sequence 5'-GAGACTAAGCCCTCC -3'(Eurogentec, Belgium). PNA-FISH was carried out by filtering 1 ml of an appropriate dilution through a 0.2 μm anodisc membrane (Whatman, UK). This was left to air dry. For the quantification of *L. pneumophila* the membrane was covered with 90% (v/v) ethanol to fix the cells and again air dried. The hybridization, washing and microscopy observation method was performed as described by [51]. For *H. pylori* quantification the membrane was covered with 4% (w/v) paraformaldehyde followed by 50% (v/v) ethanol for 10 minutes each to fix the cells and air dried. The hybridization, washing and microscopy observation method was performed as described by [21].

7.2.6 Statistical analysis

The homogeneity of variances of total number, PNA and cultivable cells and the relation between *L. pneumophila* of cells and total cells was checked by the Levene test for equality of variances using a statistical package (SPSS Inc., Chicago IL, USA). Results were subsequently compared by a one-way ANOVA followed by a Bonferroni post hoc test. Differences were considered relevant if $P<0.05$.

7.3 Results and Discussion

In a previous study several bacterial strains were isolated from heterotrophic biofilms formed on uPVC coupons in a two-stage chemostat system (Chapter 3 to 6). For the present work, the selection of the bacteria used was based on the prevalence of these isolated strains in biofilms, i.e., the strains that were always present in biofilm samples were used rather than those that were only found intermittently. Initially, the selected biofilm strains were tested for auto and co-aggregation in test tubes as described by Rickard et al. [38], either alone or with *L. pneumophila*. No aggregation was observed for the strains studied either alone or in pairs with *L. pneumophila* (results not shown).

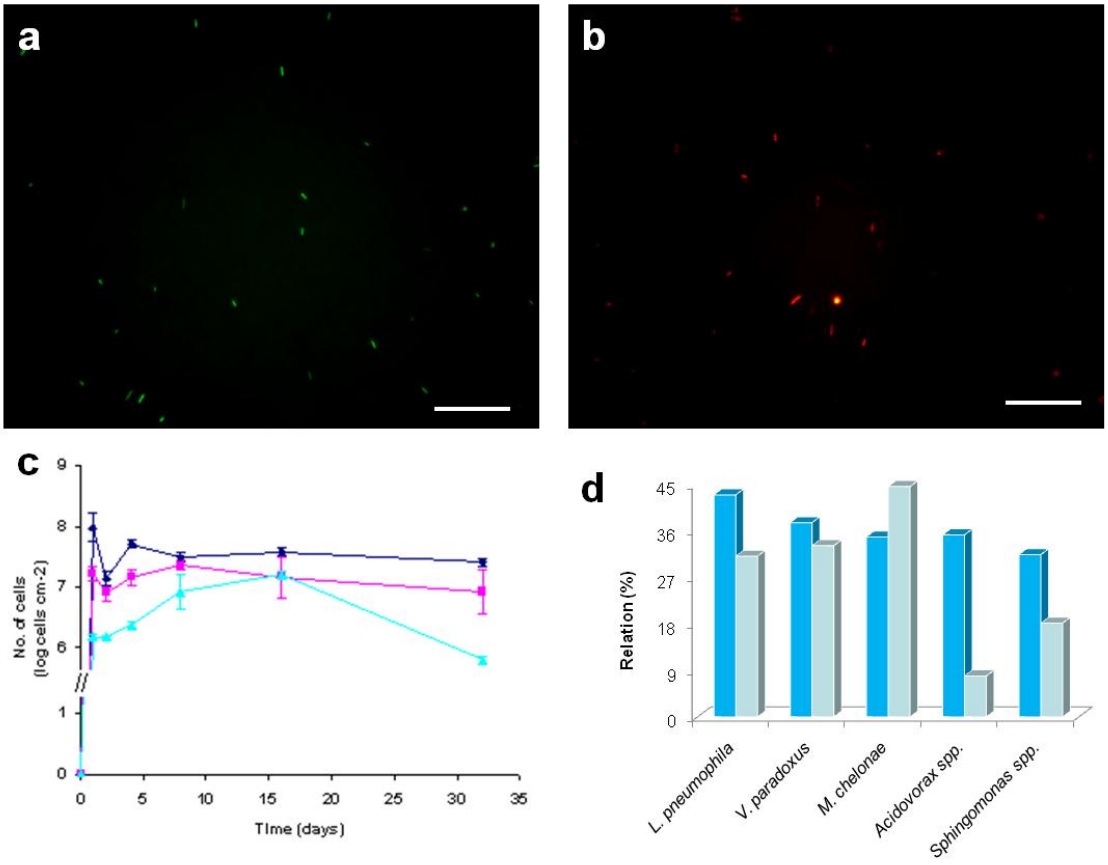

Figura 7.1. Epifluorescence microphotograph of *L. pneumophila* cells from the inoculum stained with SYTO 9 (a) and labeled by the PNA PLPEN620 probe (b). Bars represent 20 μm. (c) Variation with time in the total cell number (◆), *L. pneumophila* bound to the PNA PLPEN620 probe (■) and cultivable *L. pneumophila* (▲) present in the *L. pneumophila* pure biofilm. (d) Average of the relation between the numbers *L. pneumophila* PNA cells and total cells (turquoise bars) and relation between cultivable *L. pneumophila* and *L. pneumophila* PNA cells (bright blue bars) for the pure and dual species biofilm.

For the experiments of biofilm formation on uPVC coupons, a *L. pneumophila* inoculum containing approximately 3.7 x 10^7 cells ml^{-1} was prepared; 49% were cultivable and 50% were detected by PNA-FISH. Figures 7.1a and 1b describe cells from the inoculum stained with SYTO 9 and the PNA probe, respectively, showing the differences of the two methods used. Cells obtained from rich media agar

plates are normally considered to be in a good physiological state and the similarity in the cultivable and PNA detectable numbers, and the difference between PNA-labeled and total cells, strongly indicates that the PNA probe failed to detect cells in all metabolic states. PNA probes have been used to detect pathogens in mixed biofilms but it has not been well established if this technique can also detect non-viable cells. In fact, some authors have suggested that as selected PNA probes bind specifically to rRNA molecules, and after cellular death the content of rRNA decreases significantly, the emission of a bright signal is a good indication of a high rRNA content and viability [6, 28, 51]. The results obtained in this work corroborate that hypothesis. The inocula of the other strains had on average 75% of cultivable cells except in the case of *Mycobacterium chelonae* where the percentage was considerably lower (2.5%).

To simplify the presentation of results, only the variation with time of total cells, PNA-cells and cultivable *L. pneumophila* present in the biofilm of the control experiment are shown (Figure 7.1c). As it has been also shown before by other authors [42, 43], most of the cells attach to the uPVC surface in the first day and the numbers of total and PNA cells did not change significantly ($P>0.05$) while cultivable cells have a great increase in the first 2 weeks and decrease significantly in the two last weeks of the experiment ($P<0.05$). It has been demonstrated that *L. pneumophila* can survive in tap water for long periods without losing cultivability [35]. However, the bacterium is not able to replicate in axenic cultures in tap water or in low nutrient media but only when associated with biofilms or parasitizing amoeba species [33, 45]. Moreover, the similarity in the numbers of PNA and cultivability suggest that cells that are not cultivable also have a low ribosomal content, and hence are probably in a non-viable state. As such, the increase in the numbers of cultivable cells present in biofilms can be explained by the fact that biofilms exist in a dynamic pseudo steady-state, meaning that while portions of biofilm are constantly being sloughed off, more cells are attaching to the surface [9, 52]. If the cells that detach are preferentially non cultivable and there is attachment of cultivable cells then the numbers of cultivable cells embedded in the biofilm increase. Moreover, Rogers et al. (1992) [44] showed that *L. pneumophila* can grow as microcolonies in complex consortia biofilms [44]. Although single species biofilm cells appear to have lost cultivability, probably due to exhaustion of essential nutrients in the batch culture system, PNA numbers remained constant and suggest that cells are still viable. On the other hand, the maintenance of cultivability by some cells indicate that biofilms are a protective niche for *L. pneumophila* even in axenic culture. The fact that total *L. pneumophila* and PNA-labeled *L. pneumophila* remained constant with time indicates that there is no damage to DNA and rRNA structures along the experiment and therefore the variation of PNA numbers in mixed biofilms can be a good indicator of the variation of total *L. pneumophila* inside of those biofilms.

The numbers of *L. pneumophila* PNA cells and cultivable *L. pneumophila* when associated with other bacteria do not change significantly with time after the first day ($P>0.05$). Table 7.1 shows that the average of numbers of total cells, total PNA *L. pneumophila* and cultivable *L. pneumophila* in pure biofilms and in dual species biofilms were similar, except for the numbers of cultivable *L. pneumophila* when associated with *Acidovorax* sp. that were significantly lower ($P<0.05$). Figure 7.1d shows the percentage of numbers of PNA *L. pneumophila* in relation to total cells and the percentage of cultivable *L. pneumophila* in relation to PNA *L. pneumophila*. In the first case the percentage appears

to be similar for all the cases studied which suggests that *L. pneumophila* adhere well to uPVC surfaces, either alone or in the presence of *V. paradoxus*, *M. chelonae*, *Acidovorax* sp. and *Sphingomonas* sp. The relation between cultivable and total *L. pneumophila* is higher (although not statistically significative, P>0.95) for cells recovered from *L. pneumophila – M. chelonae* biofilm indicating that this latter strain has a small positive effect on *L. pneumophila* cultivability. In contrast, the numbers of cultivable *L. pneumophila* decreased four times when this pathogen is associated with *Acidovorax* sp. and two times when associated with *Sphingomonas* sp. This indicates that these two species have a negative impact on *L. pneumophila*, either by competition for nutrients (these two microorganisms were isolated on R2A that is a nutrient-poor defined medium, meaning that they have low nutritional requirements to grow, contrary to *L. pneumophila* which is not able to grow in absence of, for example, L-cysteine and high iron concentrations) or production of a metabolite toxic to *L. pneumophila*. Another explanation might be attributed to the structure of the biofilm. Figure 7.2 shows a 32 days-old biofilm formed by *L. pneumophila* and *L. pneumophila* associated with *Sphingomonas* sp. The biofilm formed in the presence of *Sphingomonas* sp. has a thicker structure where anaerobic zones might occur hence inducing the non-cultivable state in *L. pneumophila*. It has been demonstrated that under anaerobic conditions *L. pneumophila* loses cultivability [47]. However, the fact that the numbers quantified by the use of a PNA probe remained constant, indicate that these cells may still be viable and can recover cultivability under favorable conditions.

Table 7.1. Average of the total number of cells (quantified by the use of SYTO9), *L. pneumophila* (quantified by the PNA-FISH method) and cultivable *L. pneumophila* cells on biofilms formed by *L. pneumophila* in pure culture and *L. pneumophila* in a dual-species culture with each one of the species isolated from drinking water biofilms.

Strain on biofilm	**Total cells x 10^{-7} (cells cm^{-2})**	**PNA cells x 10^{-7} (cells cm^{-2})**	**Cultivable *L. pneumophila* x 10^{-6} (CFU cm^{-2})**
L. pneumophila	4.42	1.48	5.25
V. paradoxus	3.51	1.11	4.11
M. chelonae	4.87	1.05	4.65
Acidovorax sp.	4.12	1.59	1.05
Sphingomonas sp.	3.80	0.83	1.45

It is well known that other bacteria can influence the growth of *L. pneumophila* either in nutrient-poor environments, such as drinking water, or in rich artificial media. Toze et al. [45] have demonstrated that some bacteria commonly present in heterotrophic biofilms, such *Pseudomonas* spp. and *Aeromonas* spp., can inhibit the growth of *L. pneumophila* while Wadowsky et al. [48] demonstrated that *Flavobacterium breve* can support the satellite growth of this pathogen in BCYE without L-cysteine. A curious result was obtained by Temmerman and colleagues [44] that demonstrated that dead cells can also support the growth of this pathogen. Although the mechanisms responsible for the

influence of different microorganisms in *L. pneumophila* are unknown there is one aspect of *L. pneumophila* microbial ecology that has been already well-established: *L. pneumophila* is not able to grow in drinking water unless associated with biofilms or amoeba species ([15, 32],reviewed in [24]). Hence, the knowledge of how microorganisms affect *L. pneumophila* growth might play a key factor for the effective control of this pathogen in drinking water and requires further investigation.

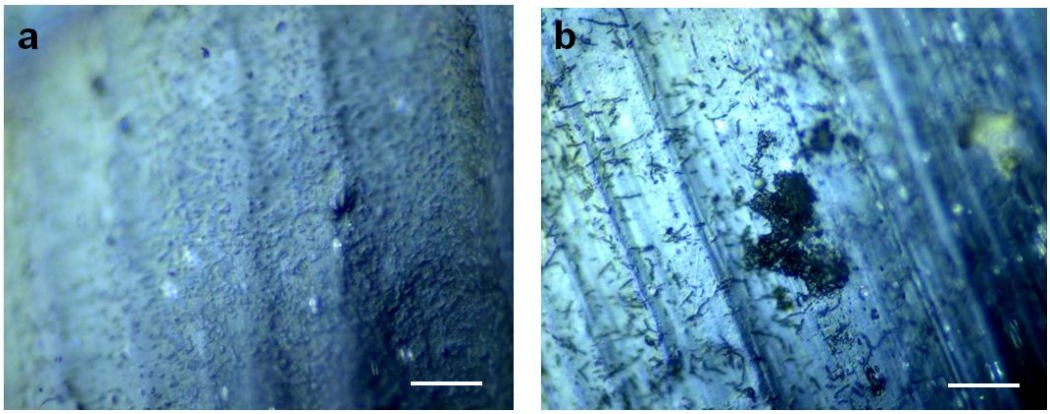

Figura 7.2. Microphotograph of a uPVC coupon visualized under EDIC microscopy covered with a 32 days-old biofilm formed by *L. pneumophila* (a) and *L. pneumophila* and *Sphingomonas* sp. (b). Bars represent 20 μm.

The same experiments were repeated using *H. pylori* instead of *L. pneumophila* and performing an extra experiment with *Brevundimonas* sp., a bacterium isolated on CBA medium from drinking water biofilms. The results in test tubes also reveal no co-aggregation with any of the bacteria. In fact, several species isolated from drinking water biofilms do not present auto-aggregation, and it has been particularly demonstrated for *Brevundimonas vesicularis*, *Acidovorax delafieldii* and *V. paradoxus* [10, 39].

In the *H. pylori* inoculum only 5.0% of the total cells were cultivable, a value similar to the value obtained by Azevedo and colleagues [2], while 29% were detected by PNA-FISH. The difference between cultivable and PNA numbers indicates that although coming from a rich medium some of the cells were already in a VBNC state. However the poor cultivability of the cells on agar media do not seem to be an obstacle to the formation of biofilm, as shown for biofilms formed after 1 and 32 days (Figure 7.3a, b and c).

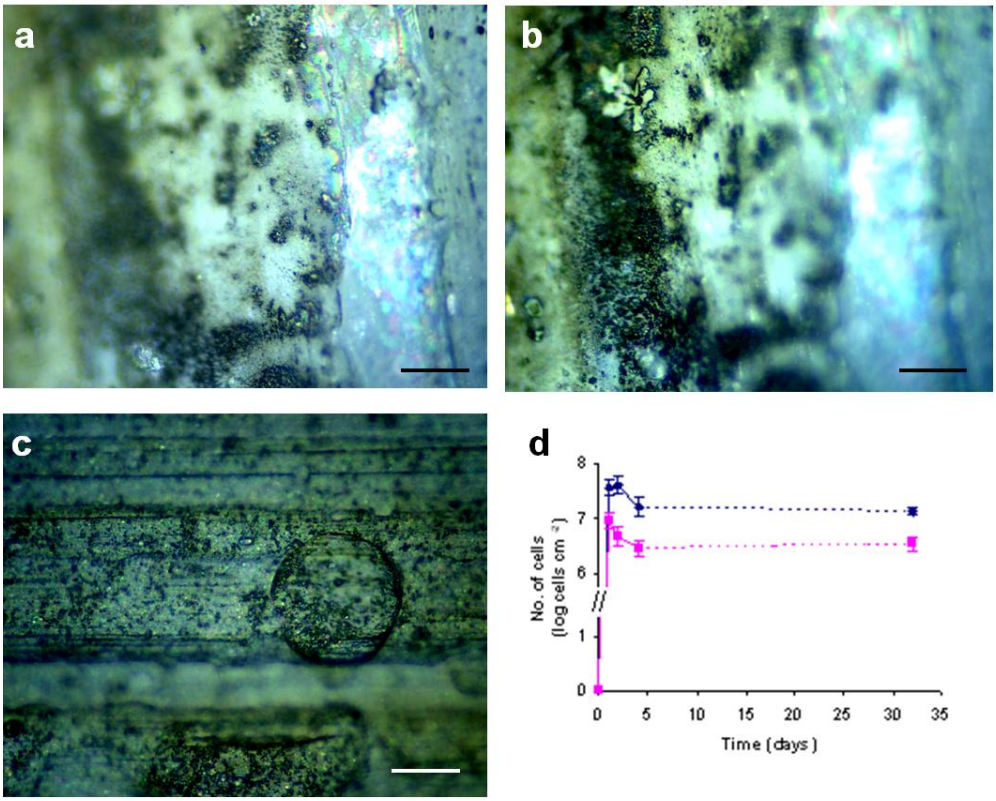

Figura 7.3. Microphotograph of a uPVC coupon visualized under EDIC microscopy covered with a 1 day-old biofilm formed by *H. pylori* in pure culture in two different visual planes bottom (a) and top (b) and 32 days-old biofilm (c). Bars represent 20 μm. (d) Variation with time in the total cell number (◆) and *H. pylori* PNA-cells (■) present in the biofilm.

Figure 7.3d shows that when in pure culture *H. pylori* adheres to the surface to form the biofilm in the first day followed by a statistically significant decrease in total cells ($P<0.05$) but only for the first 4 days. The same trend was observed for total cells and cells quantified by the PNA probe. No cultivable *H. pylori* were recovered on CBA medium, which is opposite to the Azevedo et al [1] studies, that after 24 hours there were still cultivable cells in the biofilm. This might be due to the differences in method of cell removal from the coupons, the quality of water or the type of uPVC. When the biofilm was formed in the presence of *Brevundimonas* sp. the variation with time of total cells and PNA numbers were not statistically significant ($P>0.05$). In this case due to the fast and easy growth of *Brevundimonas* on CBA medium it is not possible to conclude about the loss of cultivability of *H. pylori* when recovered from biofilms. Comparing the numbers obtained for pure *H. pylori* biofilms and biofilms grown in the presence of *Brevundimonas* sp. there was no significant difference between the numbers of *H. pylori* detected using the PNA probe (results not shown) or in the percentage between PNA and total cells numbers ($P>0.05$) which suggest that this bacterium as little or no effect on the inclusion of *H. pylori* in biofilms.

The recovery of cultivable *H. pylori* from biofilms grown in the presence of *M. chelonae* and *Sphingomonas* sp. (6.67×10^1 and 1.83×10^2 CFU cm^{-2}, respectively) suggests that these microorganisms might have a positive effect on the inclusion and survival of this pathogen in drinking

water biofilms. The ability of *H. pylori* to adaptat to different physico-chemical parameters has been studied by several authors [4, 5, 31, 33, 50] however no studies about the influence of other microorganisms on the survival of this pathogen have been found in literature except the coculture of *H. pylori* with the protozoa *Acanthamoeba castellanii* [52]. The synergetic interaction of microorganisms in biofilms is well documented and in this particular case can be the key for the survival of this microorganism in drinking water systems. More investigations should therefore be performed concerning the influence of drinking water microorganisms on *H. pylori* metabolism and survival.

When *H. pylori* cells were visualized under epifluorescence microscopy after being hybridized with the *H. pylori* specific probe, it was observed that in the inoculum the morphology of the cells was predominantly spiral while after forming biofilms the cells were mainly coccoid shaped. The coccoid shape has been demonstrated by Azevedo and coworkers [1] as an environmental adaptation of this pathogen to stress conditions and is associated to the VBNC state. It would be interesting to further investigate and verify if these cells were able to recover and cause infections in mice or guinea pigs.

This work clearly demonstrates that, even in pure culture, both pathogens can adhere to surfaces and form biofilm. *L. pneumophila* can remain cultivable for at least 32 days although less cultivable when associated with *Acidovorax* sp. and *Sphingomonas* sp. On the other hand *H. pylori* loses the cultivability in less than 24 hours except when associated with *M. chelonae* and *Sphingomonas* sp. *M. chelonae* seems to have an important effect on the cultivability of both pathogens and being a pathogen commonly found in drinking water systems [14, 18], can play an important role in disinfection procedures. Control of this mycobacteria opportunistic pathogen and other biofilm species that can have a synergetic effect on *L. pneumophila* and *H. pylori* might provide an important contribution towards the supply of safe dinking water as both *L. pneumophila* and *H. pylori* have been found to be chlorine resistant.

7.4 Acknowledgements

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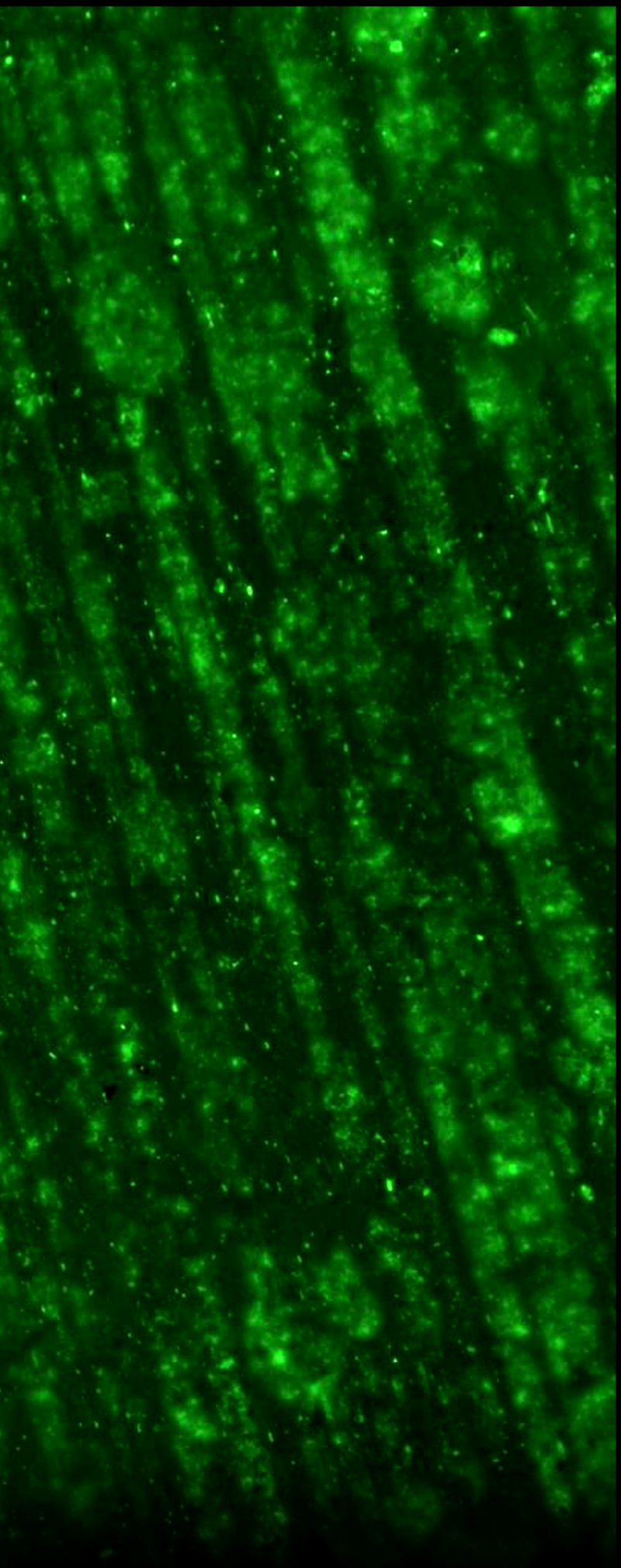

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Chapter 8

Final Conclusions and Perspectives of Work

In this chapter, the principal conclusions of this work are summarized and further work highlighted to answer questions that were not fully resolved.

8.1 Final Conclusions

The main objective was to study the influence of chlorine on *L. pneumophila* and *H. pylori* and although certain aspects of this process have been successfully clarified, many new questions have appeared.

After disinfection, residual chlorine must be provided to the final drinking water to ensure the microbiological safety of tap water. However it was shown that low concentrations of chlorine are ineffective in the control of *L. pneumophila*. It was demonstrated that after chlorination *L. pneumophila* can completely lose its cultivability, however cells remain viable and capable of causing infection in amoeba cells and consequently possibly in humans (Chapter 2). Moreover, drinking water biofilms can work as a protective niche for *L. pneumophila* resulting in a very high tolerance to different low concentrations of chlorine (Chapter 4).

With the *H. pylori* experiments similar results were obtained (Chapter 6). In the planktonic state, cells lost cultivability without losing viability and surprisingly initial dead cells disappeared after exposure to chlorine, indicating that chlorine reacts predominantly with weaker/damaged cells. The experiments with heterotrophic biofilms also showed that biofilms are a refuge for *H. pylori* in chlorinated waters and inside these structures different concentrations of chlorine have no effect on the total numbers of this pathogen.

In chapters 3 and 5, it was shown that heterotrophic biofilms support the inclusion of *L. pneumophila* and *H. pylori* in biofilms under different physico-chemical conditions. For *L. pneumophila,* shear stress and carbon concentration seems to have little effect on the concentration of *L. pneumophila* existing in drinking water biofilms, however, lower temperatures seem to favour the inclusion of this pathogen. Concerning *H. pylori*, temperature had no effect in terms of *H. pylori* numbers inside the biofilm. Differences were obtained at 15ºC for shear stress and carbon addition compared to the control. It is important to note that although the numbers of *H. pylori* obtained for both temperatures were similar, the cells were predominantly spiral shaped at 20ºC and coccoid at 15ºC which indicates that *H. pylori* adapts its shape to different environmental conditions as a survival strategy.

The fact that in suspension, cells have lost cultivability without losing viability and in biofilms cultivable cells were never recovered for the two pathogens tested, strongly supports that cultivable standard methods are an ineffective measure for controlling water quality and assessing disinfection efficiency and that alternative methods to detect VBNC, such as FISH or PCR, should be developed.

The influence of particular bacteria in biofilm formation and survival of *L. pneumophila* and *H. pylori* was also assessed (Chapter 7). The bacterium *M. chelonae* seems to have an important role in the cultivability of both pathogens while other heterotrophic bacteria decreased the cultivability of *L. pneumophila*. These results also support the theory that the lack of cultivability in biofilms could be due to the overgrowth of other heterotrophic bacteria and not only the ability of bacteria to enter a VBNC state.

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8.2 Future Work

This work has highlighted several aspects of chlorination and biofilm protection function. Nevertheless, three years and half were not sufficient to answer all the questions and hence further investigation is needed.

The development of alternative methods for the microbiological safety control of drinking water can rely on the validation of PNA probes to detect specific viable pathogens. The confirmation that PNA probes only detect viable and not total cells can be attained by the use of several methods simultaneously, such as LIVE/DEAD, direct viable count and infectivity of animal models. In the later case, for the particular pathogens studied in this work, amoeba species might prove to be a good and easier alternative to animal models.

The deleterious effects of low concentrations of chlorine on *L. pneumophila* and *H. pylori* cells is not well understood. It was attempted during this work to analyse the protein expression of both pathogens after exposure to chlorine. The method used was iTRAQ and proteins would be detected by Mass Spectrometry. This work is ongoing and will be very valuable as the comparison of proteins expressed before and after exposure to the oxidative stress of chorine can help to understand the disinfection action of chlorine and adopt better strategies to control these two pathogens.

The experiments of dual-species biofilms might also be expanded, by using other bacteria isolated from drinking water to understand the role of different microorganisms in the behaviour of *L. pneumophila* and *H. pylori*.

Finally, after validating PNA probes to detect viable cells, field studies can be performed to detect *H. pylori* in water and definitely prove that water can be a route of transmission of this pathogen, as only epidemiological studies have so far correlated water and incidence of these pathogens. And… did not John Snow suspect about cholera be a waterborne disease based on epidemiological studies? And was he not proved to be right?

Appendix I

Scientific Outputs

I.1 Accepted and Submitted papers in peer reviewed international journals

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Detection of *Helicobacter pylori* in drinking water biofilms by *fluorescence in situ hybridization*" (doi:10.1128/AEM.00827-08).

Gião, M S, Wilks, S A, Azevedo, N F, Vieira, M J and Keevil, C W. "Validation of LIVE/DEAD® to detect viable but non-cultivable *Legionella pneumophila*" (submitted).

Gião, M S, Wilks, S A, Azevedo, N F, Vieira, M J and Keevil, C W. "Incorporation of natural uncultivable Legionella pneumophila into potable water biofilms provides a protective niche against chlorination stress" (submitted).

Gião, M S, Wilks, S A, Azevedo, N F, Vieira, M J and Keevil, C W. "Comparison between standard culture and *fluorescence in situ hybridization* methods to study the influence of physico-chemical parameters on *Legionella pneumophila* survival in drinking water biofilms" (submitted).

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Resistance of *Helicobacter pylori* to chlorine in drinking water biofilms" (in preparation).

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Interaction of *Legionella pneumophila* and *Helicobacter pylori* with bacterial species isolated from drinking water biofilms" (in preparation).

I.2 Oral presentations in international conferences and meetings

Azevedo N F, Gião M S, Almeida C, Fernandes I, Keevil C W and Vieira, M J. (September 2007). "Relevance of heterotrophic biofilms on the agglomeration of H. pylori in water environments: implications for transmission". XX International Workshop on Helicobacter and related bacteria in chronic digestive inflammation, Istanbul, Turkey, 20th to 22nd September 2007.

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Influence of carbon concentration, shear stress and temperature on survival of *Legionella pneumophila* in drinking water biofilms". 4th ASM Conference on Biofilms, Québec City, Canada, 25th to 29th March 2007.

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Assessment of viable but non cultivable cells of *Helicobacter pylori* after chlorination". Sixth progress meeting SAFER, Universidade do Minho, Braga, Portugal, 7th and 8th April 2006.

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Influence of carbon concentration and shear stress on survival of *Legionella pneumophila* in drinking water biofilms". Sixth progress meeting SAFER, Universidade do Minho, Braga, Portugal, 7th and 8th April 2006.

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Assessment of Viable but non-cultivable cells of *Legionella pneumophila* after disinfection". Fifth progress meeting SAFER, Riga Technical University, Riga, Latvia, 6th and 7th October 2005.

I.3 Poster presentations in international conferences

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Resistance of *Legionella pneumophila* and *Helicobacter pylori* to chlorination in drinking water biofilms". 12th International Symposium on Microbial Ecology (ISME – 12), Cairns, Australia, 17th to 22nd August 2008.

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Influence of physico-chemical parameters on the survival of *Helicobacter pylori* in drinking water biofilms". 14th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms (CHRO), Rotterdam, The Netherlands, 2nd to 5th September 2007.

Wilks, S A, Gião, M S, Vieira, M J and Keevil, C W "Legionella pneumophila is an abundant and chlorine tolerant autochthonous member of potable water biofilms". 107th ASM General Meeting American Society for Microbiology (ASM), Toronto, Canada, 21st to 25th May 2007.

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Survival of *Legionella pneumophila* and *Helicobacter pylori* in drinking water after chlorination". 11th International Symposium on Microbial Ecology (ISME – 11), Vienna, Austria, 20th to 25th August 2006.

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Survival of VBNC *Legionella pneumophila* in drinking water following chlorine disinfection". 2nd Congress of European Microbiologists – FEMS 2006, Madrid, Spain, 4th to 8th July 2006.

Gião, M S, Azevedo, N F, Wilks, S A, Vieira, M J and Keevil, C W. "Assessment of Viable but non-cultivable cells of *Legionella pneumophila* after disinfection". WaterMicro 05, University of Wales, Swansea, UK, 4th to 9th September 2005.