ADVERTIMENT. La consulta d'aquesta tesi queda condicionada a l'acceptació de les següents condicions d'ús: La difusió d'aquesta tesi per mitjà del servei TDX (www.tesisenxarxa.net) ha estat autoritzada pels titulars dels drets de propietat intel·lectual únicament per a usos privats emmarcats en activitats d'investigació i docència. No s'autoritza la seva reproducció amb finalitats de lucre ni la seva difusió i posada a disposició des d'un lloc aliè al servei TDX. No s'autoritza la presentació del seu contingut en una finestra o marc aliè a TDX (framing). Aquesta reserva de drets afecta tant al resum de presentació de la tesi com als seus continguts. En la utilització o cita de parts de la tesi és obligat indicar el nom de la persona autora.

ADVERTENCIA. La consulta de esta tesis queda condicionada a la aceptación de las siguientes condiciones de uso: La difusión de esta tesis por medio del servicio TDR (www.tesisenred.net) ha sido autorizada por los titulares de los derechos de propiedad intelectual únicamente para usos privados enmarcados en actividades de investigación y docencia. No se autoriza su reproducción con finalidades de lucro ni su difusión y puesta a disposición desde un sitio ajeno al servicio TDR. No se autoriza la presentación de su contenido en una ventana o marco ajeno a TDR (framing). Esta reserva de derechos afecta tanto al resumen de presentación de la tesis como a sus contenidos. En la utilización o cita de partes de la tesis es obligado indicar el nombre de la persona autora.

WARNING. On having consulted this thesis you're accepting the following use conditions: Spreading this thesis by the TDX (www.tesisenxarxa.net) service has been authorized by the titular of the intellectual property rights only for private uses placed in investigation and teaching activities. Reproduction with lucrative aims is not authorized neither its spreading and availability from a site foreign to the TDX service. Introducing its content in a window or frame foreign to the TDX service is not authorized (framing). This rights affect to the presentation summary of the thesis as well as to its contents. In the using or citation of parts of the thesis it's obliged to indicate the name of the author

UNIVERSITAT POLITÈCNICA
DE CATALUNYA

Reclaimed water use pilot scale practices in the Catalonian region. Viability quantitative PCR for microbial water quality monitoring

This thesis is presented for the degree of Doctor of philosophy by

Mariana Fittipaldi Gustavino

Directed by

Jordi Morató

Verónica Rajal

Doctorado en Sostenibilidad, Tecnología y Humanismo
Cátedra UNESCO de Sostenibilidad

2014

A mi familia

"Water should be judged by its quality; not its history"

(Dr.Lucas Van Vuuren)

Summary

Water is absolutely essential for the life of present and future generations. It is a basic, indispensable and potentially renewable resource. The last means that the resource will be renew depending on whether its exploitation rate does not exceed its regeneration rate, thus a sustainable water use is required. Nowadays, lack of water, both in terms of quality and quantity, is a serious worldwide problem, as well as, an indication that the use of this resource has not been sustainable. Water availability is affected by both natural and anthropogenic factors, including climate change, pollution, natural water source overexploitation, and technological factors. Likewise, water demand does not remain constant over time; it increases together with population increases, varies with changes in preferences and social values, and increases or decreases with technological innovation.

The United Nations has designated the period from 2000 to 2015 as the International Decade for Action "Water for Life" in order to achieve the Millennium Development Goals, which involve achieving halving the number of people without access to safe drinking water by 2015, and to stop unsustainable exploitation of water sources. It implies the need of an integrated water management that considers the multiplicity of utilities and functions that water provides.

Reclaimed water use is an essential element in the integrated water resources management. Wastewater treatment and reuse are activities that increase the water capital without depleting the natural hydric resources. Reclaimed water can be used in different applications depending on its quality, thus reducing the potable water demand, and allowing for water natural sources regeneration. Furthermore, the regenerated effluent is kept out of the surface waters and groundwater preventing their quality deterioration, and consequently reducing environmental degradation.

Despite large advances in wastewater treatment, waterborne diseases still pose a major worldwide threat to public health. Consequently, the use of reclaimed water usually requires more stringent monitoring procedures than when "good-quality" water is used (groundwater and drinking water) in order to guarantee its microbiological quality over time. Improving water microorganisms detection techniques is essential to help optimizing all steps involving treatment and use of reclaimed water, and thus to encourage its use in key sectors such as agriculture and industry. Quantitative polymerase chain reaction (qPCR) has the potential to be one of the quickest and useful methods currently available for microbial detection and quantification, and it could be a useful tool for water quality monitoring and control.

Research is needed to reduce persistent uncertainty about the potential adverse effects that the use of reclaimed water may have on human health and the environment, with the ultimate goal of increasing confidence in reuse practices and public acceptance of them. In order to contribute to these purposes, this dissertation work was performed along two main intertwined lines of research: (1) the study of qPCR methods as water microbiological quality monitoring tools; and (2) the study of microbiological colonization associated to reclaimed water use at pilot scale practices using culture and qPCR techniques.

An initial approximation to molecular methods was performed optimizing different qPCR methods, followed by a deep work performed with viability qPCR technique aiming at improving it, especially to be used for environmental sample analysis.

A qPCR method to detect *Legionella pneumophila* in water samples was optimized and it resulted to be a powerful screening tool for monitoring *Legionella pneumophila* in hot water and cooling water samples, allowing for fast and reliable results.

Viability qPCR technique using ethidium monoazide (EMA) and propidium monoazide (PMA) was validated using cultured and environmental samples. The addition of a pre-treatment step to the sample analysis to inhibit the amplification of DNA from membrane-damaged cells has been used in combination with qPCR to detect live cells of *Legionella pneumophila*, *Helicobacter pylori*, *Bacteroides* spp., *Escherichia coli*, infective bacteriophage T4, and viable *Acanthamoeba castellani* trophozoites and cysts through experimental work performed during the course of this dissertation. Treatment with viability dyes profits from the fact that it is easy to perform, it is compatible with existing technology and it does not significantly increase the time to results. Consequently, as it was observed throughout this work, viability qPCR is a highly valuable technique for a wide range of applications. However, some limitations of this technique were identified. The incomplete suppression of the dead cell amplification signals was observed in many cases leading to false-positive results. Thus, viable cell quantification may be affected by the presence of high levels of dead cells. In part these limitations can be minimized by choice of experimental variables and conditions adequate for a particular sample. However, protocol optimization for each sample and each microorganism is not always easy to achieve.

Considering the possible technique limitations, a strategy suggesting the performance of three independent qPCR reactions on identical sample aliquot was proposed in this work to minimize the influence of false-positive and false-negative results. This new approach, which has been validated using environmental samples, provides a realistic estimate of the number of live cells, and also provides a better understanding of microbial dynamics in complex samples, such as reclaimed water. In the absence of robust and reliable procedures, and keeping in mind that in microbiology it is very difficult have accurate results on viability assessment due to the heterogeneous nature of microbial life, the concept as outlined in this dissertation is interesting as a future research direction.

Two different pilot systems were constructed and studied from a microbiological point of view to evaluate different reclaimed water uses, such as industrial and agricultural applications. In the case of industrial water reuse, the analysis was carried out using cooling water systems. The effect of water origin in the *Legionella* colonization of cooling towers was verified using demonstration units. Moreover, the bacterial colonization of a cooling tower pilot system with *in situ* disinfection was monitored. In the case of agricultural water reuse, the potential health risk associated with vegetable crops irrigation using reclaimed water was evaluated. To this end, four different water sources were used and extensive microbiological analysis of water and vegetable tissue samples were performed. In both cases, microbiological analyses were conducted by culture, qPCR, and viability qPCR techniques.

Under the studied conditions of both pilot systems, the use of secondary-treated wastewater was associated with higher bacterial load levels compared to those levels observed when tertiary-treated wastewater was used. The herein used reclaimed water with some kind of disinfection treatment behaved equivalent to untreated well water with regard to the risk of *Legionella* colonization and biological growth in general. These results emphasize the important role that disinfection treatments play on water reuse. Application of on-site disinfection at the end-use point or/and at the storage step is highly recommended. Especially, considering the great variability in microbial quality that different reclaimed water batches can have and the loss of quality that water may suffer during distribution and storage steps. Moreover, if end-point disinfection is used, good quality secondary-treated wastewater is capable of being used, as it was demonstrated in this work. The importance of the application of one or two washing steps when vegetables are irrigated with reclaimed water was also observed in this work, this is especially important for products that are consumed raw.

In conclusion the analyzed conditions present a favorable scenario for the use of reclaimed water, as long as it complies with a minimum quality standards established in the Spanish Royal Decree 1620/2007.

In these pilot studies the viability qPCR technique showed, in general, the same microbial colonization dynamic and similar pollution levels than culture, therefore the same but faster conclusions can be achieved when this technique is used. However, some DNA amplification

inhibition problems were observed when vegetables samples were analyzed. Thus, technical and procedures improvements are required and should be addressed in future studies.

The work developed throughout this dissertation helps to reduce persistent uncertainty in relation to the potential adverse effects that may encompass the use of reclaimed water on human health by demonstrating that the reclaimed water use, under suitable and controlled conditions, does not entail greater microbiological contamination when compared to well water. It also provides more light on the use of qPCR and viability qPCR techniques as tools for control and monitoring of water quality in order to address effective microbial pollution prevention. Quantitative PCR is essential for detection of specific pathogens and/or pathogens which can be present in very low numbers. The fact that it may also provide information on the viability and infectivity of microorganisms, as demonstrated in this dissertation, makes it a very powerful tool for rapid and reliable monitoring of water quality, as well as, it allows for quick response time for decision making. Therefore, it could be a useful tool for the implementation of microbial quality control programs.

Additionally, the herein developed and applied triple qPCR approach might help to reduce overestimation of bacterial viability in complex matrices like wastewater samples, particularly when protocol optimization will be difficult to be performed.

As established Dr. Lucas Van Vuuren *"water should be judged by its quality; not its history"* [1]and viability qPCR is a good tool to achieve this.

[1] Howe, C., Mitchell, C. 2012. Water sensitive cities. IWA Publishing, London, UK, pp. 113. Available in http://books.google.es/. Last access 12/04/2013.

Resumen

El agua es absolutamente imprescindible para la vida de las generaciones presentes y futuras. Es un recurso básico, insustituible y potencialmente renovable. Un recurso potencialmente renovable lo será realmente si su tasa de explotación no supera su tasa de regeneración, lo cual requiere que el uso de dicho recurso sea sostenible. La falta de agua, tanto en términos de calidad como de cantidad, es actualmente un problema grave a nivel mundial y un indicador de que su uso no ha sido sostenible. Su disponibilidad se ve afectada tanto por factores naturales como antropogénicos, incluyendo el cambio climático, la contaminación, la sobreexplotación de las fuentes naturales de agua y factores tecnológicos. Así mismo, la demanda hídrica tampoco se mantiene constante; incrementa con los aumentos de población, varía con los cambios en las preferencias y valores sociales y se acrecienta o decrece con la innovación tecnológica.

Las Naciones Unidas han designado el período que va del 2000 al 2015 como la Década Internacional para la Acción "Agua para la vida", con el fin de cumplir algunos de los Objetivos de Desarrollo del Milenio. Entre las metas propuestas para el año 2015 hallamos la de reducir en un cincuenta por ciento la cantidad de gente sin acceso a agua potable y la de frenar la explotación insostenible de las fuentes acuíferas. Esto implica la necesidad de una gestión integral del agua, teniendo en cuenta la multiplicidad de utilidades y funciones que ofrece este recurso.

La reutilización de agua regenerada constituye un elemento clave en la gestión integral de los recursos hídricos. El tratamiento del agua residual y su posterior reutilización son actividades que permiten incrementar el capital agua sin agotar el recurso natural. Siempre que su calidad lo permita, el agua regenerada puede utilizarse en diferentes aplicaciones, reduciendo de este modo la demanda de agua potable y otorgando un mayor tiempo de regeneración a las fuentes naturales. Además, el efluente se mantiene fuera de las corrientes acuíferas superficiales y subterráneas evitando que la calidad de las mismas se vea deteriorada, lo que disminuye la degradación del medio ambiente.

A pesar de los grandes avances alcanzados en el tratamiento de aguas residuales, las enfermedades transmitidas por el agua siguen representando una amenaza mundial importante para la salud pública. En consecuencia, para garantizar su calidad microbiológica a lo largo del tiempo, el uso de este tipo de recurso suele requerir un monitoreo o control de calidad más estricto que el de otros tipos considerados como de buena calidad (como por ejemplo el agua de pozo y el agua potable). La mejora de las técnicas de detección de microorganismos en agua es esencial para optimizar el tratamiento y utilización del agua regenerada, y poder así fomentar su uso en sectores claves como la agricultura y la industria. La reacción en cadena de la polimerasa en tiempo real, conocida como qPCR por sus siglas en inglés (quantitative polymerase chain reaction), es uno de los métodos más rápidos y útiles disponibles actualmente para la detección y cuantificación de microorganismos y podría constituir una herramienta útil para el seguimiento y control de la calidad del agua.

La investigación es necesaria para reducir la incertidumbre persistente sobre los posibles efectos adversos que puede tener el uso de agua regenerada en la salud humana y en el ambiente, con el objetivo de incrementar la confianza en las prácticas de reutilización y la aceptación pública de las mismas. Para contribuir a estos fines, en esta tesis se trabajó en dos líneas principales, pero entrelazadas, de investigación: (1) el estudio de la técnica de qPCR como herramienta rápida y eficaz para el control y monitoreo de la calidad microbiológica del agua, y (2) el estudio de la colonización microbiológica asociada con el uso de agua regenerada en prácticas a escala piloto usando técnicas de microbiología convencional, cultivo en placa, y técnicas de biología molecular, qPCR.

Inicialmente se realizó una primera aproximación a los métodos moleculares poniendo a punto distintos protocolos de qPCR, para continuar con un profundo trabajo realizado en el desarrollo y mejora de la técnica de qPCR de viabilidad para el análisis de muestras ambientales.

Se logró optimizar un método de qPCR para detectar Legionella pneumophila en muestras de agua, el cual resultó una herramienta útil para el monitoreo de Legionella pneumophila en muestras de agua caliente y de torres de refrigeración en tanto permitió obtener resultados rápidos y fiables.

La técnica de qPCR de viabilidad fue validada utilizando monoazida de etidio (Ethidium Monoazide EMA) o monoazida de propidio (Propidium Monoazide, PMA) tanto para el análisis de muestras de cultivos puros como de muestras ambientales. A lo largo del trabajo experimental realizado durante el desarrollo de la tesis, la adición de un pretratamiento al análisis de la muestra para inhibir la amplificación del ADN proveniente de células con su membrana dañada se ha utilizado en combinación con la qPCR con el fin de detectar células vivas o infecciosas de Legionella pneumophila, bacteriófago T4, trofozoítos y quistes de Acanthamoeba castellani, Helicobacter pylori, Bacteroides spp. y Escherichia coli. El pretratamiento de las muestras usando estos compuestos químicos es fácil de realizar, compatible con la tecnología existente y no aumenta significativamente el tiempo de obtención de los resultados por lo que, como se observó a lo largo de este trabajo, la qPCR de viabilidad es una técnica de gran valor para un amplio rango de aplicaciones.

El desarrollo de la investigación sirvió asimismo para identificar algunas limitaciones de esta técnica que pueden conducir a la obtención de resultados no del todo correctos. Una de ellas es que la cuantificación de células viables podría verse afectada por la presencia de elevados niveles de células muertas, ya que la supresión de la señal de amplificación para dichas células no se alcanzó por completo. Las limitaciones observadas pueden minimizarse, en parte, mediante una elección de las variables y condiciones experimentales adecuadas para una muestra en particular. Sin embargo, la optimización de un protocolo para un tipo de muestra o microorganismo particular no siempre es fácil de alcanzar por lo que se propuso una estrategia que sugiere la realización de tres reacciones de qPCR independientes para minimizar la influencia de los resultados falsos positivos y falsos negativos. Este nuevo enfoque, el cual se validó usando muestras ambientales, brinda una estimación más realista del número de células vivas presentes en una muestra y ofrece una mejor comprensión de la dinámica microbiana en muestras complejas, como las de agua regenerada. En ausencia de procedimientos robustos y fiables y teniendo presente que en microbiología, debido a la naturaleza heterogénea de la vida microbiana, es muy difícil obtener resultados precisos cuando la viabilidad celular es evaluada, el concepto que ofrece este nuevo enfoque resulta interesante como una línea de investigación futura.

Dos sistemas pilotos fueron construidos y estudiados para evaluar el uso del agua regenerada en diferentes aplicaciones, como la industria y la agricultura, desde un punto de vista microbiológico. En el caso de estudio de la reutilización de agua en la industria, el análisis se llevó a cabo usando sistemas de refrigeración de agua. Se verificó el efecto del origen del agua en la colonización por Legionella de torres de refrigeración utilizando unidades demostrativas, y se monitoreó la colonización bacteriana de un sistema piloto de torre de refrigeración con desinfección in situ. En el caso de estudio de la reutilización de agua en la agricultura, se evaluó el riesgo sanitario potencial asociado al riego de cultivos de hortalizas con aguas regeneradas. Para esto se utilizaron cuatro fuentes de agua diferentes y se realizó el análisis microbiológico de muestras hídricas y de tejido vegetal. En ambos casos, para el análisis microbiológico de las muestras se utilizaron las técnicas de cultivo en placa, qPCR, y qPCR de viabilidad.

Bajo las condiciones de estudio de los dos sistemas pilotos, el uso del agua regenerada con tratamiento secundario se asoció a niveles más altos de carga bacteriana en comparación con los niveles observados para el agua regenerada con tratamiento terciario. El agua regenerada con algún tipo de tratamiento de desinfección mostró un comportamiento equivalente al del agua de pozo sin tratar con respecto al riesgo de colonización por Legionella y al crecimiento biológico en general. Estos resultados señalan el importante rol

que juegan los tratamientos de desinfección en la reutilización de agua. De los mismos se desprende también la recomendación del uso de tratamientos de desinfección en el punto de consumo y/o durante la etapa de almacenamiento, sobre todo teniendo en cuenta la gran variabilidad en la calidad microbiana que los diferentes lotes de agua regenerada pueden tener, y la pérdida de calidad que puede sufrir este recurso durante las etapas de distribución y almacenamiento. Por otra parte, si se utiliza desinfección en el punto de uso, el agua regenerada con tratamiento secundario de buena calidad es susceptible de ser utilizada, tal como se demostró en los casos analizados en esta tesis. Los estudios realizados destacaron también la importancia de la aplicación de una o dos etapas de lavado cuando se riegan las verduras con agua regenerada, especialmente en los cultivos de aquellos vegetales que se consumen crudos.

En conclusión, las condiciones analizadas presentan un escenario favorable para la reutilización de agua regenerada, siempre y cuando se cumpla con unos parámetros mínimos de calidad como los establecidos en el Real Decreto Español 1620/2007.

En estos casos de estudio la técnica PMA- qPCR mostró, en general, la misma dinámica en la colonización microbiana y similares niveles de contaminación que la técnica de cultivo en placa, por lo que se observa que pueden lograrse los mismos resultados, pero más rápidos, cuando la primera técnica es utilizada. Sin embargo, algunos problemas de inhibición fueron observados en el análisis de muestras vegetales y requieren que la técnica PMA- qPCR y los procedimientos sean mejorados en futuras investigaciones.

El trabajo desarrollado en esta tesis contribuye a reducir la incertidumbre persistente en relación con los efectos adversos potenciales que puede tener el uso de agua regenerada en la salud humana mediante la demostración de que el uso de este recurso, en condiciones adecuadas y controladas, no implica un mayor riesgo de contaminación microbiológica en comparación con el agua de pozo.

Este trabajo aporta asimismo más luz sobre el uso de las técnicas de qPCR y qPCR de viabilidad como herramientas de control y monitorización de las aguas para la prevención eficaz de la contaminación microbiológica. La qPCR resulta esencial para la detección de patógenos específicos y/o que pueden estar presentes en concentraciones bajas. El que además pueda aportar información sobre la viabilidad e infectividad de los microorganismos la convierte en una herramienta muy potente que permite un monitoreo rápido y fiable de la calidad del agua, y contribuye a una mejor toma de decisiones en los casos en que sea necesario, por lo que podría ser una herramienta útil para la implementación de programas de control de calidad microbiológica.

Adicionalmente, la modificación propuesta en esta tesis para la qPCR de viabilidad podría ayudar a reducir la sobre-estimación del número de células vivas en matrices complejas como las aguas residuales, sobre todo cuando la optimización del protocolo sea difícil de realizar.

Como sostiene el Dr.Lucas Van Vuuren *"el agua debe ser juzgada por su calidad, y no por su historia"*[2] y la qPCR de viabilidad es una buena herramienta para lograr este cometido.

[2] Howe, C., Mitchell, C. 2012. Water sensitive cities. IWA Publishing, London, UK, pp. 113. Disponible en http://books.google.es/. Último acceso 04/12/2013.

Resum

L'aigua és absolutament imprescindible per a la vida de les generacions presents i futures. És un recurs bàsic, insubstituïble i potencialment renovable. Un recurs potencialment renovable ho serà si la seva taxa d'explotació no supera la taxa de regeneració, la qual cosa requereix que l'ús d'aquest recurs sigui sostenible. La falta d'aigua, tant en termes de qualitat com de quantitat, és actualment un problema greu a nivell mundial i un indicador que el seu ús no ha estat sostenible. La disponibilitat d'aigua es veu afectada tant per factors naturals com antropogènics, incloent el canvi climàtic, la contaminació, la sobreexplotació de les fonts naturals d'aigua i factors tecnològics. Així mateix, la demanda d'aigua tampoc es manté constant; s'incrementa amb els augments en la població, varia amb els canvis en les preferències i valors socials, i augmenta o disminueix amb la innovació tecnològica.

Les Nacions Unides han designat el període de l'any 2000 al 2015 com la Dècada Internacional per a l'Acció "Aigua per la vida" amb la finalitat de complir alguns dels Objectius del Mil·lenni, com són especialment la reducció a la meitat de la quantitat de gent sense accés a aigua potable per a l'any 2015 i frenar l'explotació insostenible de les fonts d'aigua. Això implica la necessitat d'una gestió integral de l'aigua, tenint en compte la multiplicitat d'utilitats i funcions que l'aigua ofereix.

La reutilització d'aigua regenerada és un element clau en la gestió integral dels recursos hídrics. El tractament de l'aigua residual i la seva posterior reutilització són activitats que permeten augmentar el capital d'aigua sense esgotar el recurs natural. L'aigua regenerada pot usar-se en diferents aplicacions sempre que la seva qualitat així ho permeti, reduint d'aquesta manera, la demanda d'aigua potable, i atorgant un major temps de regeneració a les fonts naturals. A més, l'efluent es manté fora dels corrents d'aigües superficials i subterrànies, evitant que la qualitat de les mateixes es vegi deteriorada i, en conseqüència, disminueix la degradació del medi ambient.

Malgrat els grans avenços aconseguits en el tractament d'aigües residuals, les malalties transmeses per l'aigua segueixen representant una important amenaça mundial per a la salut pública. En conseqüència, per garantir la seva qualitat microbiològica al llarg del temps, l'ús d'aquest tipus d'aigua sol requerir un monitoreig o control de qualitat més estricte que quan s'utilitzen altres tipus considerats com de bona qualitat (aigua de pou i aigua potable). La millora de les tècniques de detecció de microorganismes a les aigües és essencial per optimitzar el tractament i l'ús de l'aigua regenerada, especialment en sectors claus com l'agricultura i la indústria. La reacció en cadena de la polimerasa en temps real, coneguda com qPCR per les seves sigles en anglès (quantitative polymerase chain reaction), té el potencial de ser un dels mètodes més ràpids i útils disponibles actualment per a la detecció i quantificació de microorganismes; i podria ser una eina útil per al seguiment i control de la qualitat de l'aigua.

La investigació és necessària per reduir la incertesa persistent sobre els possibles efectes adversos que pot tenir l'ús d'aigua regenerada en la salut humana i en l'ambient, amb l'objectiu d'incrementar la confiança en les pràctiques de reutilització i l'acceptació pública de les mateixes. Per poder contribuir a aquestes finalitats, en aquesta tesi es va treballar en dues línies principals, però entrellaçades, de recerca: (1) l'estudi de la tècnica de qPCR com a eina ràpida i eficaç per al control i monitoreig de la qualitat microbiològica de l'aigua, i (2) l'estudi de la colonització microbiològica associada amb l'ús d'aigua regenerada en pràctiques a escala pilot, utilitzant tècniques de microbiologia convencional, cultiu en placa, i tècniques de biologia molecular, qPCR.

Inicialment es va realitzar una primera aproximació als mètodes moleculars posant a punt diferents protocols de qPCR, per continuar amb un profund treball realitzat en el desenvolupament i millora de la tècnica de qPCR de viabilitat, per a l'anàlisi de mostres ambientals.

Es va aconseguir optimitzar un mètode de qPCR per detectar *Legionel·la pneumophila* en mostres d'aigua, el qual va resultar ser una eina útil pel monitoreig de *Legionel·la pneumophila* en mostres d'aigua calenta i de torres de refrigeració, permetent obtenir resultats ràpids i fiables.

La tècnica de qPCR de viabilitat va ser validada utilitzant monoazida d'etidi (Ethidium Monoazide EMA) o monoazida de propidi (Propidium Monoazide, PMA) tant per a l'anàlisi de mostres de cultius purs com a mostres ambientals. Al llarg del treball experimental realitzat durant el desenvolupament de la tesi, l'addició d'un pretractament a l'anàlisi de la mostra per inhibir l'amplificació de l'ADN provinent de cèl·lules amb la seva membrana danyada s'ha utilitzat en combinació amb la qPCR per detectar cèl·lules vives o infeccioses de *Legionel·la pneumophila*, bacteriòfag T4, trofozoïts i cists d'*Acanthamoeba castellani*, *Helicobacter pylori*, *Bacteroides* spp., i *Escherichia coli*. El pretractament de les mostres usant aquests compostos químics és fàcil de realitzar, compatible amb la tecnologia existent i no augmenta significativament el temps d'obtenció dels resultats. Per tant, com es va observar al llarg d'aquest treball, la qPCR de viabilitat és una tècnica de gran valor per a un ampli rang d'aplicacions. No obstant això, s’han identificat algunes limitacions d'aquesta tècnica en el desenvolupament dels estudis que componen aquesta tesi. La quantificació de cèl·lules viables podria veure's afectada per la presència d'alts nivells de cèl·lules mortes, ja que el senyal d'amplificació per a aquestes cèl·lules no es va suprimir per complet i es van obtenir resultats falsos positius. Les limitacions observades poden minimitzar-se, en part, mitjançant una elecció dels paràmetres i condicions experimentals adequades per a una mostra en particular. No obstant això, l'optimització d'un protocol per a un tipus de mostra o microorganisme particular, no sempre és fàcil d'aconseguir.

Tenint en compte les possibles limitacions de la tècnica, es va proposar una estratègia que suggereix la realització de tres reaccions de qPCR independents per minimitzar la influència dels resultats falsos positius i falsos negatius. Aquest nou enfocament, el qual es va validar usant mostres ambientals, brinda una estimació més realista del nombre de cèl·lules vives presents en una mostra i ofereix una millor comprensió de la dinàmica microbiana en mostres complexes, com l'aigua regenerada. En absència de procediments robusts i fiables i, tenint en compte que en microbiologia, a causa de la naturalesa heterogènia de la vida microbiana, és molt difícil obtenir resultats precisos quan la viabilitat cel·lular és avaluada, el concepte d'aquest nou enfocament és interessant com a línia d'investigació futura.

Dos sistemes pilots van ser construïts i estudiats per avaluar l'ús de l'aigua regenerada en diferents aplicacions, com la indústria i l'agricultura, des d'un punt de vista microbiològic. En el cas d'estudi de la reutilització d'aigua a la indústria, l'anàlisi es va dur a terme usant sistemes de refrigeració d'aigua. Es va verificar l'efecte de l'origen de l'aigua en la colonització per *Legionel·la* de torres de refrigeració utilitzant unitats demostratives, i es va monitorejar la colonització bacteriana d'un sistema pilot de torre de refrigeració amb desinfecció in situ. En el cas d'estudi de la reutilització d'aigua en l'agricultura, es va avaluar el risc sanitari potencial associat al reg de cultius d'hortalisses amb aigües regenerades. Per a això es van utilitzar quatre fonts d'aigua diferents i es van realitzar les anàlisis microbiològiques de mostres d'aigua i teixit vegetal. En tots dos casos, l'anàlisi microbiològica de les mostres es va realitzar usant cultiu en placa, qPCR, i qPCR de viabilitat.

Sota les condicions d'estudi dels dos sistemes pilots, l'ús de l'aigua regenerada amb tractament secundari es va associar a nivells més alts de càrrega bacteriana en comparació als nivells observats per a l'aigua regenerada amb tractament terciari. L'aigua regenerada amb algun tipus de tractament de desinfecció va mostrar un comportament equivalent al de l'aigua de pou sense tractar pel que fa al risc de colonització per *Legionel·la* i el creixement biològic en general. Aquests resultats ressalten l'important rol que els tractaments de desinfecció juguen en la reutilització d'aigua. Dels mateixos també es desprèn la recomanació de l'ús de tractaments de desinfecció en el punt de consum i/o durant l'etapa

d'emmagatzematge. Sobretot, tenint en compte la gran variabilitat en la qualitat microbiana que els diferents lots d'aigua regenerada poden tenir, com es va observar en aquest treball, i la pèrdua de qualitat que l'aigua pot sofrir durant les etapes de distribució i emmagatzematge. D'altra banda, si s'utilitza desinfecció en el punt d'ús, l'aigua regenerada amb tractament secundari de bona qualitat és susceptible de ser utilitzada com es va demostrar en els casos analitzats en aquesta tesi. Els estudis realitzats també van mostrar la importància de l'aplicació d'una o dues etapes de rentat quan es reguen les verdures amb aigua regenerada, especialment en els cultius que es consumeixen crus.

En conclusió, les condicions analitzades presenten un escenari favorable per a la reutilització d'aigua regenerada, sempre que es compleixi amb uns paràmetres mínims de qualitat com els establerts en el Reial Decret 1620/2007.

En aquests casos d'estudi la tècnica PMA- qPCR va mostrar, en general, la mateixa dinàmica en la colonització microbiana i similars nivells de contaminació que la tècnica de cultiu en placa, per tant les mateixes però més ràpides conclusions poden aconseguir-se quan aquesta tècnica és utilitzada. No obstant això, alguns problemes d'inhibició van ser observats en l'anàlisi de mostres vegetals i requereixen que la tècnica i els procediments siguin millorats en futurs treballs.

El treball desenvolupat en aquesta tesi contribueix a reduir la incertesa persistent en relació amb els efectes adversos potencials que pot tenir l'ús d'aigua regenerada en la salut humana, mitjançant la demostració que l'ús d'aigua regenerada, en condicions adequades i controlades, no implica un major risc de contaminació microbiològica en comparació de l'aigua de pou.

Aquest treball també aporta més llum sobre l'ús de les tècniques de qPCR i qPCR de viabilitat com a eines de control i seguiment de les aigües per a la prevenció eficaç de la contaminació microbiològica. La qPCR és essencial per a la detecció de patògens específics i/o que poden estar presents en concentracions baixes. El fet que a més pugui aportar informació sobre la viabilitat i infectivitat dels microorganismes, com s'ha demostrat en aquesta tesi, la converteix en una eina molt potent que permet un monitoreig ràpid i fiable de la qualitat de l'aigua, així com també contribueix a una millor presa de decisions en els casos que sigui necessari. Per tant, podria ser una eina útil per a la implementació de programes de control de qualitat microbiològica.

Addicionalment, la modificació proposada en aquesta tesi per la qPCR de viabilitat podria ajudar a reduir la sobre-estimació del nombre de cèl·lules vives en matrius complexes com les aigües residuals, sobretot quan l'optimització del protocol sigui difícil de realitzar.

Com va establir el Dr.Lucas Van Vuuren "l'aigua ha de ser jutjada per la seva qualitat, i no per la seva història"[3] i la qPCR de viabilitat és una bona eina per aconseguir-ho.

[3] Howe, C., Mitchell, C. 2012. Water sensitive cities. IWA Publishing, London, UK, pp. 113. Disponible en http://books.google.es/. Últim accés 04/12/2013.

Acknowledgments

Even when I many time thought this moment would not come, I finally here trying to express in few pages my gratitude to all those people who in any way made possible that this chapter of my life end happily. This dissertation work could not be performed without their contributions and support.

Perhaps these pages are the the most beautiful but difficult to write because it is not easy to find the proper words for expressing gratitude for all those moments, chats, silences, mistakes, learning, team work, "scientific" discussions which strengthened the feel of doing science, coffees, lunches, laughs, outstretched hands that often became friendly hands, support and incentives to follow through and be tenacious, and those physical or cyber hugs received at the right time. All the support I was given along this long journey let me reach not only an intellectual challenge, but also a great personal growth.

I would like to first and foremost thank my thesis supervisors, Dr. Jordi Morato and Dr. Verónica Rajal, who gave me the opportunity to be part of a research group and to do science. Jordi, thanks for teaching me to develop my abilities and fend by myself. Thanks for your confidence also. Vero, thank you very much for bringing me in first contact with Jordi, and above all for not forget me in the way, for your time, for knowledge and experience shared, for continuous improvement search, and especially for believing in me at all times.

I gratefully acknowledge to the *Comissionat per a Universitats i Recerca del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya i del Fons Social Europeu* for the financial support received through the pre-doctoral fellowships FI program and for the Catalunya outside research stays fellowship program (BE 2008).

Especial thanks to Francesc Codony who dedicated so many hours of his precious time; for his experience shared, his opinions and valuable comments. Francesc, thanks for your patience and your interest to interpreting my ideas, for your help and guidence on this research journey. Thanks for driving me into the microbiology world and for sharing your craving to know. Thank you for inspire me and keep me motivated, for your kind consideration, support and trust.

I would also like to thank Dr. Anne Camper for opening her "home doors" at the *Center for Biofilm Engineering* and for made me feel part of her research group during the 4 months I stayed at the Montana State University.

I cannot thank to scientific community, especially those which belong to my areas of work, for their hard work and contributions that have been a great contribution during my research. Particularly thanks to Andreas Nocker whom I had the honour and the great opportunity to work and share opinions.

I am also grateful to the research groups that have collaborated with us performing research projects. Thanks CRESCA (Centre de Recerca en Seguretat i Control Alimentari) and IRTA (Institut de Recerca i Tecnologia Agroalimentària) teams for their collaboration and facilitate good teamwork. Especial thanks to Carmen Biel and the technicians for give me your support and your smiles during the experimental work conducted in the IRTA field.

Thanks to all technicians and colleagues who helped me, and eased the experimental work.

I am deeply gratefully to my beautiful people, those I knew here and also those from my beloved Argentina. The people list is long, and that makes me happy. Regrettably, I can not mention all of them but I want let they know that they are always present in my mind and my heart, and that they are the driving force behind all what I do.

Anna, Barbara, Sonia, Sandreta and Gemma, thank you for being my mates in this journey. Nothing would have been the same without you around. You know how difficult was to get here, and I thankfully you made it easier. Thanks for worked hand to hand with me, sharing of experiences and knowledge, and for the positive support. For made lighter the gray days and good days even better. For accepting me as I am, and help me to be better. For your friendly hands so important for me. For opening us the doors of your houses, for "lend us" family and friends, for showing us Catalunya and introducing us to Catalan country life. And also thanks for letting me act as a friend ☺.

Thanks to Fidel and Curro for doing enjoyable days.

Thanks Gem and Luis for doing nice and less hard my stay in Bozeman, and thanks for the patience in the laboratory work.

Thanks Gustavo, Ruth, Alex, Elaine, Angeles, Carolina, Cecilia, Fede, Danielita, Blady, Paulina, Kamelia, Teresa, Facundo, Rodrigo, Irene, the Ales, Bet, Mar, Marta, Nico, Edrisi, José Miguel, and Rodolfo for the nice moments, talks and smiles that you gave me. I have really enjoyed my time together with you.

Thanks Vero, Vale, Romi, Mer, Letu and Estelita for your friendship maintained and strengthened over the years and despite the distance.

Mani, Jaime, Gaby, July, Nico, and Elenita thanks for welcomed with open arms when we began this adventure of living away from home, the continuous company, and be near family.

I want to express my deepest gratitude to those who are my roots, my history, my guide, my references, “my beautiful and generous bay”, my all, my FAMILY. Thanks to them, despite being so far away and so close, they are always supporting me.

Martina, Diego, Pau, and Laia, I am grateful that circumstances have made you were close us, and that we have build our beautiful family nest here. Nothing had been the same without you.

Matilde, María Sara, Martina, Clara, Matías and Marcos, thanks for being my friend-brothers, you are ones of the most important people in my life. Ramiro, Diego, Mily, María Ana, and Vistas my extensive thanks because you are my extended brothers. It is wonderful that you are part of the family; I think I could not have better brothers’ in-law. Thanks also for giving me the opportunity to be the aunt of those beautiful little ones - Manu, Pau, Mica, Fede and Benjamín - that fill me with life, innocence, freshness, and who many times remember me the essentials of life.

Bulke, Ani, Ruthy, and Judy thanks for accepting me, adopt me, love me and to be family. I am very happy and grateful for this second family that I have. Bulke thanks for your endless desire to help, to teach, and for trying to become easier some things in the lab. Ani thanks for sharing me your bacteriology knowledge and your desire to always learn more.

Especially thanks to those beautiful people, my parents, who have given me your life, your effort and your time, and that even in the distance encouraging me to finish this work. Thank you. Thanks for being what you are, for founding our wonderful family, for helping and guiding me to be who I am.

Thanks Aaron for you endless support. For your help and contributions, listening and teaching me. Because you did this journer easier, thanks for your patience, for enduring my fatigue, boredom and melancholy. For encouraging me to finish this work. Thanks above all for being my friend, my soul mate in the life journey with all that those implies.

Valentina, you have been a major motivation to finish this dissertation. Thanks for filling me with lot of love being so little and for everything you will teach me.

Agradecimientos

Aunque pensé en alguna ocasión que este momento no llegaría, aquí me encuentro finalmente tratando de plasmar en unas páginas mi gratitud a todo y todos aquellos que, de una manera u otra, hicieron posible que este capítulo de mi vida se cierre felizmente.

Quizás sean las páginas más lindas pero más difíciles de escribir, pues es difícil encontrar las palabras propicias para expresar los sentimientos y el agradecimiento por todos esos momentos, charlas, silencios, errores, aprendizajes, trabajos compartidos, discusiones "científicas" en las que esas ganas de seguir haciendo ciencia salieron fortalecidas; cafés, comidas, risas, manos tendidas que muchas veces se convirtieron en amigos, apoyo y alicientes para seguir adelante y ser constante, y esos abrazos reconfortantes, físicos o cibernéticos, que llegaron en los momentos oportunos. Todo eso posibilita que a lo largo de este camino, además de haber alcanzado un logro intelectual, haya conseguido un gran crecimiento personal.

Quiero agradecer a mis directores de tesis, el Dr. Jordi Morató y la Dra. Verónica Rajal, la oportunidad que me brindaron de formar parte de un grupo de investigación y de hacer ciencia. Jordi, gracias por ayudarme a desarrollar y explotar mis capacidades y a valerme por mí misma. Gracias también por tu confianza. Vero, muchas gracias por el primer contacto para llegar a España, y sobre todo por no olvidarme en el camino. Gracias por tu tiempo, por compartir tus conocimientos, por tu búsqueda de la mejora continua y por creer en mí en todo momento.

No puedo dejar de agradecer al Comissionat per a Universitats i Recerca del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya i del Fons Social Europeu por el soporte financiero recibido a través del programa de becas pre-doctorales FI y del programa de becas para estancias de investigación fuera de Catalunya (BE 2008).

Un enorme agradecimiento a Francesc Codony por regalarme su tiempo, su experiencia, sus opiniones y sus valiosos comentarios. Gracias Francesc por tu paciencia para comprender mis ideas y por creer que yo tenía algo que aportar, por saber leerme, por tu ayuda, y por ser mi guía en este camino de la investigación. Gracias por sumergirme en el mundo de la microbiología y por compartir conmigo tus ansias de saber, conocer e investigar. Gracias por la buena consideración y por tenerme en cuenta siempre, por tu apoyo y tu confianza.

Me gustaría también agradecer a la Dra. Anne Camper por abrirme las puertas de "su casa" en el Center for Biofilm Engineering y por hacerme sentir parte de su grupo de investigación durante los cuatro meses que estuve de estancia en la Montana State University. Gracias a ella y a su gente.

No puedo dejar de reconocer a la comunidad científica, especialmente a aquella perteneciente a mis áreas de trabajo, por su constante trabajo y contribuciones que han sido un gran aporte durante mi trabajo de investigación. Particularmente me gustaría agradecer a Andreas Nocker, con quien tuve el honor y la gran oportunidad de trabajar y compartir ideas.

Gracias también a los grupos de investigación que han colaborado con nosotros en la realización de algunos proyectos de investigación. Gracias a la gente del CRESCA (Centre de Recerca en Seguretat i Control Alimentari) y del IRTA (Institut de Recerca i Tecnologia Agroalimentària) por su colaboración y por facilitar el buen trabajo en equipo. Gracias especialmente a Carmen Biel y a sus técnicos por brindarme su ayuda y sonrisas durante los trabajos experimentales realizados en el IRTA de Torre Marimón.

Gracias a los técnicos y compañeros que me ayudaron facilitándome el trabajo experimental.

Un “gracias totales” a mi gente linda, la que conocí e hice mía aquí o durante el doctorado, y a la de mi Salta y mi Argentina queridas. La lista es larga, inmensa, y eso me hace feliz. No podré mencionar a todos pero quiero que sepan que los tengo presentes siempre y que son el motor de todo lo que hago.

Anna, Bárbara, Sonia, Sandreta y Gemma: gracias por ser mis compañeras de ruta en esta aventura. Nada hubiera sido lo mismo sin ustedes cerca. Ustedes son las personas que más saben cuánto ha costado llegar hasta aquí, gracias por hacerlo más fácil. Por trabajar mano a mano conmigo, por el intercambio de experiencias y de conocimientos. Por hacer los días grises más claros, y los días buenos todavía mejores. Por aceptarme como soy y por ayudarme a ser mejor. Por la mano amiga tan linda e importante para mí, por acogernos y abrirnos las puertas de sus casas, por “prestarnos” la familia y los amigos, por enseñarnos Catalunya y las costumbres catalanas. Por escuchar. Y, también, por dejarme ejercer de amiga ☺.

Gracias a mis chicos lindos de la Escuela de Óptica, Fidel y Curro, por hacer los días más amenos.

Gracias Gem y Luis por hacer más linda y menos dura mi estancia en Bozeman, y por la paciencia en el laboratorio.

Gracias Gustavo, Ruth, Alex, Elaine, Ángeles, Carolina, Cecilia, Fede, Danielita, Blady, Paulina, Kamelia, Teresa, Facundo, Rodrigo, Irene, los Ales, Bet, Mar, Marta, Nico, Edrisi, José Miguel y Rodolfo por los lindos encuentros, momentos, charlas y sonrisas que me regalaron.

Gracias Vero, Vale, Romi, Mer, Letu y Estelita por la amistad mantenida y reforzada a lo largo de los años y a pesar de las distancias, y por el aguante que eso implica ☺.

Mani, Jaime, Gaby, July, Nico y Elenita gracias por habernos recibidos con los brazos abiertos cuando iniciamos esta aventura de vivir lejos de casa, por la compañía continua, y por ser familia cercana.

Gracias a aquellos que son mi raíz, mi historia, mi guía, mi ejemplo, mis referentes, mi cable a tierra, mi bahía linda y generosa, mi todo, mi Familia. Gracias a ellos, que a pesar de estar tan lejos y tan cerca, siempre están en mí y para mí.

Martina, Diego, Pau y Laia, agradezco que las circunstancias hayan permitido tenerlos cerca, y que hayamos podido construir nuestro hermoso nido familiar aquí. Nada hubiese sido lo mismo sin ustedes.

Matilde, María Sara, Martina, Clara, Matías y Marcos, gracias por ser mis hermanos- amigos y por estar siempre cerca a pesar de la distancia. Ustedes son uno de los regalos más hermosos que me dio la vida. Ramiro, Diego, Mily, María Ana y Vistas gracias extensivas porque son mis hermanos extensivos. Es hermoso que sean parte de la familia, creo que no me podrían haber tocado mejores cuñados/as. Gracias también por regalarme la oportunidad de ser tía de esos locos bajitos tan maravillosos -Manu (ya no tan bajito), Pau, Mica, Fede y Benjamín- que me llenan de vida, de inocencia, de frescura, y que muchas veces me han recordado lo esencial de la vida.

Bulke, Ani, Judy y Ruthy gracias por aceptarme, adoptarme, quererme y ser familia. Estoy muy feliz y agradecida por esta segunda familia que me tocó en suerte. Bulke, gracias por tus infinitas ganas de ayudar, enseñar e intentar facilitarme algunas cosas en el laboratorio. Ani, gracias por compartir, entre mate y mate, tus conocimientos de bacteriología y tus ganas de conocer y de aprender siempre más.

Qué decirles a mis padres, a esas personas hermosas que me brindaron su vida, su esfuerzo y su tiempo, y que, aún en la distancia, me han empujado a terminar este trabajo. Gracias. Gracias por ser lo que son, por haber fundado nuestra maravillosa familia, por haberme ayudado y guiado para ser esta personita que soy ahora. Por confiar y creer en mí. Y por lograr estar siempre cerca, aquí conmigo, a pesar de la distancia.

Gracias Aarón por ayudarme siempre, por leer todos mis trabajos, por los aportes, por escucharme, por enseñarme, por facilitarme las tareas, por las discusiones, por la paciencia,

por aguantar el cansancio, el desgano, la melancolía, por animarme a seguir y por pincharme para terminar “la tesis”. Gracias, sobre todo, por ser mi amigo del alma y mi compañero de viaje, con todo lo que eso implica.

Y gracias Valentina, porque has sido una motivación muy importante para terminar “nuestra” tesis. Gracias por llenarme de amor desde tan pequeñita y por todo lo que me enseñarás.

Table of contents

Summary IV
Acknowledgments XIII
List of figures XXI
List of tables XXIII
List of pictures XXIV
Chapter 1 Introduction 1.1
1.1. Problem statement 1.1
1.1.1 . Status of water resources in the world 1.1
1.1.2 . Water resources uses 1.3
1.1.3 . New water culture 1.4
1.2. Needs for research 1.6
1.3. Objectives and overview of the dissertation 1.8
1.4. References 1.9
Chapter 2 Background 2.1
2.1. Water reclamation 2.1
2.1.1 . Definitions and historical development 2.1
2.1.2 . Wastewater treatment 2.2
2.2. Health aspects of reclaimed water use 2.3
2.2.1 . Chemical agents 2.3
2.2.2 . Biological agents 2.4
2.3. Reclaimed water uses 2.8
2.3.1 . Type of reclaimed water uses 2.8
2.3.2 . Reclaimed water use for agricultural irrigation 2.9
2.3.3 . Reclaimed water use for industrial process 2.10
2.3.4 . Reclaimed water use in the world 2.12
2.3.5 . Use of reclaimed water in Spain 2.13
2.3.6 . Reclaimed water use in Catalonia 2.15
2.4. Reclaimed water regulations and guidelines 2.15
2.4.1 . Reclaimed water regulations in Spain 2.16
2.5. Water quality monitoring 2.18
2.5.1 . Culture techniques 2.19
2.5.2 . Molecular methods 2.20
2.6. Reference 2.25
Chapter 3 Quantitative PCR using SYBR Green for detection of *Legionella pneumophila* in water samples 3.1
3.1. Introduction 3.2

3.2. Materials and Methods 3.3
3.2.1 . Water samples and culture 3.3
3.2.2 . Sample concentration and DNA extraction 3.3
3.2.3 . Quantitative PCR optimization 3.3
3.2.4 . *Legionella pneumophila* DNA standard 3.4
3.2.5 . Quantitative PCR assays 3.5
3.2.6 . Statistical analyses 3.5
3.3. Results 3.6
3.3.1 . Quantitative PCR optimization 3.6
3.3.2 . Standard curve 3.6
3.3.3 . Comparison between results from qPCR and *Legionella pneumophila* cultures 3.7
3.3.4 . Chi-Square Test (X^2) 3.9
3.4. Discussion 3.10
3.5. Conclusions 3.11
3.6. References 3.12
Chapter 4 Evaluation of *Legionella* colonization in cooling tower demostration systems when reclaimed water is used 4.1
4.1. Introduction 4.2
4.2. Materials and Methods 4.4
4.2.1 . *Legionella* colonization in cooling water demonstration units using different water sources. 4.4
4.2.2 . *Legionella* colonization of a lab-scale cooling tower using *in situ* disinfection 4.9
4.3. Results 4.11
4.3.1 . *Legionella* colonization in cooling water demonstration units using different water sources. 4.11
4.3.2 . *Legionella* colonization of a lab-scale cooling tower using *in situ* disinfection. 4.15
4.4. Discussion 4.19
4.5. Conclusions 4.23
4.6. References 4.23
Chapter 5 Difficulties and optimization strategies in the quantification of live *Legionella pneumophila* by viable qPCR in the presence of dead cells 5.1
5.1. Introduction 5.2
5.2. Materials and Methods 5.3
5.2.1 . Microorganism and medium 5.3
5.2.2 . Killing conditions 5.3
5.2.3 . PMA treatment 5.4
5.2.4 . Nucleic acid extraction 5.4
5.2.5 . Quantitative PCR 5.4
5.2.6 . Experiments 5.4
5.2.7 . Differential stain of live and dead cells on original stock of live cells and original stock of dead cells 5.5
5.3. Results 5.6
5.4. Discussion 5.10

5.5. Conclusions.....5.12
5.6. References.....5.13
Chapter 6 An approach to provide an improved interpretation of viable cells level estimation by qPCR.....6.1
6.1. Introduction.....6.2
6.2. Materials and Methods.....6.4
6.2.1 . Experimental assays.....6.4
6.2.2 . Sample processing.....6.5
6.2.3 . PMA treatment.....6.5
6.2.4 . DNA extraction and quantitative PCR quantification.....6.6
6.3. Results.....6.7
6.3.1 . *Legionella pneumophila* determination.....6.8
6.3.2 . *Bacteroides spp.* determination.....6.9
6.3.3 . *Escherichia coli* determination.....6.11
6.4. Discussion.....6.14
6.5. Conclusion.....6.15
6.6. References.....6.16
Chapter 7 Agricultural reuse of reclaimed water: a preliminary microbial study by using culture and viable qPCR methods.....7.1
7.1. Introduction.....7.2
7.2. Materials and Methods.....7.3
7.2.1 . Experimental design.....7.3
7.2.2 . Irrigation water quality monitoring.....7.4
7.3. Results.....7.9
7.3.1 . Irrigation water quality monitoring.....7.9
7.3.2 . Vegetable microbiological quality.....7.12
7.4. Discussion.....7.18
7.5. Conclusion.....7.22
7.6. References.....7.23
Chapter 8 General conclusions and recommendations.....8.1
8.1. General conclusions.....8.2
8.2. Recommendations.....8.4
8.2.1 . Reclaimed water.....8.4
8.2.2 . Viability qPCR techniques.....8.5
8.3. References.....8.5

List of figures

Figure 1.1. (A) Water availability per person. (B) Water demand and population increments (UNDP, 2006)........1.2

Figure 1.2. Water availability in the world and forecast for 2025 (Engelman et al., 2000). 1.3

Figure 3.1. A. Standard curve for the mip (▲) and dot (□) genes........3.6

Figure 3.2. Correlation between the results obtained by SYBR Green based qPCR method and those obtained by the culture isolation technique for *Legionella pneumophila* (Lp). 3.8

Figure 3.3. Melting curve and agarose gel electrophoresis for *Legionella pneumophila* using primers *mip* and *dot* as target........3.9

Figure 4.1. Design scheme of the cooling tower demonstration units........4.5

Figure 4.2. Standard curve for *Legionella pneumophila* (Lp) cells (CFU/mL) 4.11

Figure 4.3. Physico-chemical quality of outlet water from demonstration unit. 4.13

Figure 4.4. Microbial colonization of the different demonstration units, in terms of total aerobic bacteria load. 4.13

Figure 4.5. Mean *Legionella pneumophila* load values for the different demonstration units obtained by qPCR technique 4.14

Figure 4.6. *Legionella pneumophila* colonization of the different demonstration units, analyzed by qPCR technique. 4.15

Figure 4.7. Total aerobic colonization (planktonic and biofilm cells) of the model cooling system, determined by culture technique. 4.16

Figure 4.8. *Legionella pneumophila* colonization of the model cooling system, for water samples determined by both, culture and PMA-qPCR technique........4.16

Figure 4.9. *Legionella pneumophila* colonization of the model cooling system for biofilm samples determined by both, culture and PMA-qPCR technique........4.17

Figure 4.10. Total aerobic colonization of both, system and storage tanks, for water samples by culture technique. 4.18

Figure 4.11. Colonization of the model cooling system in terms of aerobic biofilm bacteria by culture technique........4.19

Figure 5.1. Standard curves for *Legionella pneumophila* live and dead cells with and without 50 μM PMA treatment. 5.7

Figure 5.2. Effect of the presence of dead cells on the quantitative detection of live cells from a lived/dead mixed sample. 5.7

Figure 5.3. Influence of dead *Legionella pneumophila* cells in the quantification of live *Legionella pneumophila* cells. 5.8

Figure 5.4. Concentration of total cells, live and dead, detected by v-qPCR from a mixture of known live and dead cell concentrations. 5.9

Figure 5.5. Effect of dead cell concentration on the quantitative detection of *Legionella pneumophila* live cells by fluorescence using LIVE/DEAD® BacLight TM Bacterial Viability Kit 5.9

Figure 6.1. Different theoretical possibilities when photoactivable intercalating dyes are used for v-qPCR........6.3

Figure 6.2. Presence of *Legionella pneumophila* in reclaimed water samples measured using three qPCR assays: rqPCR, PMA treatment and qPCR (v-qPCR), killing isopropanol treatment and v-qPCR (k&v-qPCR)........6.8

Figure 6.3. Determination of *Bacteroides* spp. reclaimed water samples measured using three qPCR assays: qPCR, PMA treatment and qPCR (v-qPCR), and killing heat treatment and v-qPCR (k&v-qPCR)..6.9

Figure 6.4. Presence of *Bacteroides* spp. in reclaimed water with chlorine disinfection *in situ* samples..6.10

Figure 6.5. Presence of *Bacteroides* spp. in reclaimed water with photocatalysis disinfection in situ samples measured using three qPCR assays: real-time PCR (qPCR), PMA treatment and qPCR (v-qPCR), killing heat treatment and v-qPCR (k&v-qPCR).............................6.11

Figure 6.6. Determination of *Eschericha coli* in reclaimed water samples measured using three qPCR assays: qPCR, PMA treatment and qPCR (v-qPCR), and killing heat treatment and v-qPCR (k&v-qPCR)..6.12

Figure 6.7. Presence of *Escherichia coli* in reclaimed water with chlorine disinfection *in situ* samples..6.13

Figure 7.1. Secondary-treated wastewater disinfection pilot system. 7.5

Figure 7.2. Vegetable sampling and analysis process. 7.8

Figure 7.3. Total coliform plate counts for lettuce samples irrigated with waters of four different qualities. 7.13

Figure 7.4. *Escherichia coli* plate counts for wash water samples of carrots irrigated with waters of four different qualities...7.15

Figure 7.5. Total coliform plate counts for wash water and vegetable samples of carrots irrigated with waters of four different qualities. 7.16

Figure 7.6. Total coliform bacterial plate counts from vegetable and wash water samples of string beans irrigated with waters of four different qualities..7.17

List of tables

Table 2.1. Biological agents potentially present in untreated wastewater.......................2.5

Table 2.2. Requirements of wastewater treatment for reuse in different industrial uses (Asano and Visvanathan, 2001) 2.12

Table 2.3. Reclaimed water use in Spain distributed among regions. Adapted from EPSAR (2012). 2.14

Table 2.4. Type of reclaimed water uses in Spain (CEDEX, 2008)...............................2.15

Table 2.5. Quality requirements for industrial and agricultural uses of reclaimed water according to the RD 1620/2007. ....2.17

Table 3.1. *Legionella* and non-*Legionella* bacteria used in qPCR 3.4

Table 3.2. Contingency table for *dot* and *mip* real-time PCR 3.9

Table 4.1. Physico-chemical and microbiological characteristics of different water effluents. 4.12

Table 4.2. Microbiological quality, determined in terms of fecal bacteria, of the outlet water from the demonstration units. 4.14

Table 4.3. Physico-chemical and microbiological characteristics of the reclaimed water used in each experimental assay. 4.16

Table 6.1. Oligonucleotide primers and probes used in to detect *Legionella pneumophila mip* gene, *Bacteriodes* spp. 16S rRNA gene, and *Escherichia coli uidA* gene. 6.7

Table 6.2. Live *Escherichia coli* cell number reduction during photoactivation treatment. 6.14

Table 7.1. Physico-chemical quality of the different water sources. 7.9

Table 7.2. Microbiological water quality determined by culture**.** 7.10

Table 7.3. *Escherichia coli* quantification in secondary treated wastewater by qPCR with and without PMA...7.11

Table 7.4. Total aerobic bacteria in lettuce samples irrigated with water of different quality. 7.14

Table 7.5. Water quality requirements for reclaimed water use for raw eaten vegetable irrigation (RD 1620/2007)..7.18

List of pictures

Picture 4.1.A.Details of the demonstration unit tank: electric heaters and water level sensor. B. Deposit with the electric heaters and the submersible pump responsible for water recirculation in the system. C. Tank vent, pump to control the level in the system tank, and its connections. 4.5

Picture 4.2. A. Detail of the removable column's top connections and construction. B. Baskets used to contain the column's packing properly. **C.** Packing used in the column of each demonstration unit. 4.6

Picture 4.3. Cooling tower demonstrative units located to the Blanes WWTP. The large water reservoirs for each water type are located in the back. 4.6

Picture 4.4. *Legionella* spp. plate counts. A: *Legionella* colonies can be perfectly identified and quantified. B: the results are undeterminable due to non-*Legionella* bacteria overgrowth. C: Some *Legionella* colonies can be identified but the results are non-quantifiable. 4.15

Picture 4.5. Appearance of the water samples from both, storage tank (on the right) and the system tank (on the left). 4.17

Chapter 1

Introduction

1.1. Problem statement

Water is a vital resource. People need clean water and sanitation to protect health and maintain their dignity. In addition, water also preserves the ecological and production systems which human sustenance is based on. Water, in many ways, determines all aspects of human development.

In spite that the access to safe water has been recognized as a human right by the United Nations General Assembly (World Health Organization and UNICEF, 2012), at least one billion people are deprived of the right to safe drinking water, and more than two billion people lack access to adequate sanitation (Loutfy, 2011). Furthermore, a recent study has shown that each year about 801,000 children younger than 5 years of age die as the result of diarrheal diseases (Liu et al., 2012). Nowadays, clean freshwater is no longer guaranteed, even in “water-rich” countries. Therefore, preservation of water resources is very important, and the need of a more integrated management becomes of paramount importance aiming at its sustainable use.

1.1.1. Status of water resources in the world

Human kind is almost completely surrounded by water. The human body is made up of 70% water, and planet Earth biosphere has more mass of water than of land (71% of land area is water). Water is a finite, invaluable, and irreplaceable resource and its use has limitations because not all water in the Earth is available for human use.

Regarding water on Earth, 97% is salt water and it encompasses the oceans and seas. The remaining 3% is freshwater of which two thirds are locked up in glaciers and Artic and Antarctic icecaps; and of the remaining third, almost all of it (98%) is below our feet as groundwater and about 2% is contained in rivers and lakes (Bouwer, 2002). These latest

sources are not only the water we are most familiar with, but also the sources of most of the water that is used in our everyday lives. This water is called freshwater "available" for human consumption, and its availability is subject to various factors such as accessibility for capture and subsequent use, economic costs, and environmental and social costs among others.

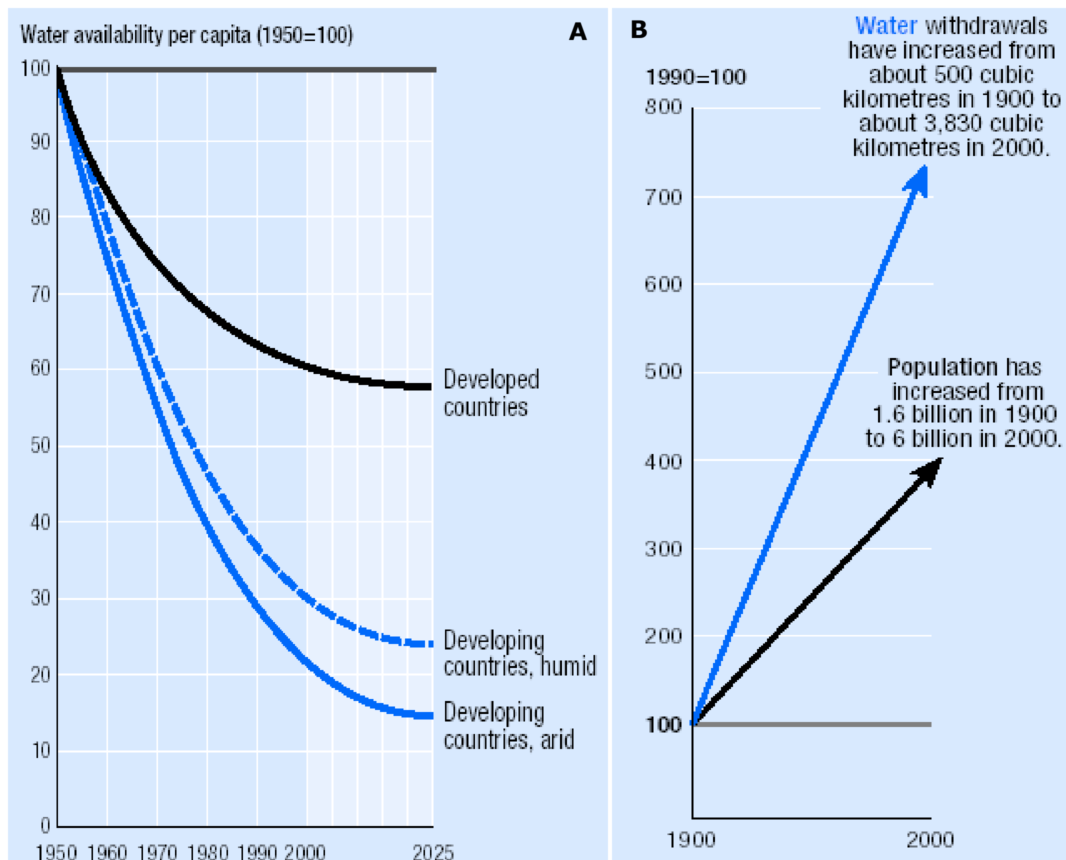


Figure 1.1. (A) Water availability per person. (B) Water demand and population increments (UNDP, 2006).

Continued population growth reduces the availability of water resources per person (Figure 1.1 (A)). But, as shown in Figure 1.1 (B), increased water consumption in the world in the last century was not proportional to population growth, indicating the existence of other factors influencing this consumption increment. One of the most important factors is the growth and continued economic development that leads to changes in lifestyle and the development of new technologies. Moreover, a negative impact of climate change on freshwater resources is also expected.

Another issue to be considered is that the “available surface freshwater” must remain in the rivers and streams to safeguard the environmental integrity. The rate at which this protection should occur depends on the specific ecological limit of each river/lake below which it is expected that the system will be degraded.

If the supply of freshwater was equally distributed, it would be more than adequate to sustain the world’s rapidly growing population as well as the ecological integrity. However, because both the freshwater supply and the world’s population are unevenly distributed, there are many regions which suffer from severe water shortages. Figure 1.2 shows the current water availability in the world and its forecast for the year 2025. The water stress

index serves as a rough indicator for the pressure exerted on water resources. It is a ratio between the water withdrawals and the freshwater available for human consumption. However, it is worth of mention that different water uses bring about dissimilar stress. Values of less than 10%, indicate that the water stress is low; a water stress value between 10 and 20% indicates that water availability is becoming a constraint on development, while a water stress index above 20% is supposed to necessitate comprehensive management efforts to balance supply and demand, and actions to resolve conflicts among competing water uses (Organization for Economic Cooperation and Development (OECD), 2003). The most affected regions according this index are the Arab States and Sub-Saharan Africa (UNDP, 2006). But looking at future projections, the outlook is grim, as it shows an accelerated expansion of the water shortage in the world. Estimates indicate that water stress and water scarcity in the world are expected to increase by 26% and by 4%, respectively (Figure 1.2).

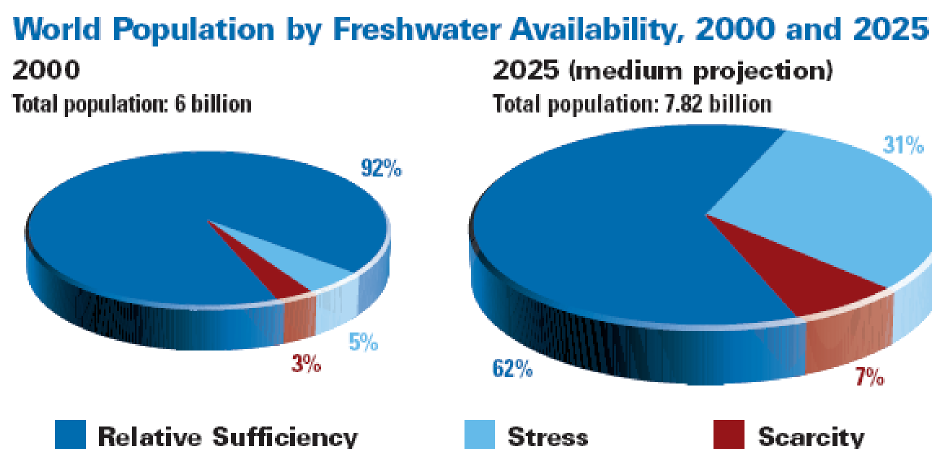


Figure 1.2. Water availability in the world and forecast for 2025 (Engelman et al., 2000).

Europe has plenty of water resources compared to other regions of the world, and water has long been considered as an inexhaustible public commodity. However, this position has changed in the last decades by the growth of water stress, both in terms of water scarcity and quality deterioration (Bixio et al., 2006). According to Hochstrat and Wintgens (2003), about half of the European countries, representing almost 70% of the population, are facing water stress issues today. Spain is among them, showing a water stress index higher than 20% (Hochstrat and Wintgens, 2003).

While in developing countries the problem linked to lack of safe drinking water supply and improved sanitation is tremendous; within the so-called developed countries the infrastructures required to tackle supply and sanitation are fairly well built-up (coverage of a 98% in 2002, World Health Organization and UNICEF, 2005). Therefore, in these regions, concerns related to the water cycle focus on water stress caused by anthropogenic influences such as industrial, agricultural or human dwelling activities and the whole range of its impact on health associated, environmental and socio-economical issues (Gernjak, 2006).

1.1.2. Water resources uses

There are three main sectors requiring water, namely: agriculture, industry, and public supply. Globally, it is estimated that 70% of the freshwater consumption is devoted to

agricultural activities, 20% to industry, and the remaining 10% to domestic use[1]. However, these percentages vary according to the economic development achieved by each country (UNDP, 2006).

Proportionately, in the last fifty years water consumption in industrial and public sectors has grown more than water consumption in agriculture sector (UNDP, 2006). This may be due to increasing urbanization - "*in 1960, one in three people lived in a city; today, almost half the people live in cities and, as it is predicted, by 2030 over 60% of the world population will live in urban areas*" [2] -, the raise in living standards, extension of life expectancy, and economic growth and industrial development, mainly in developed countries.

Besides the fresh water uses above mentioned, others non-extractive (*in situ*) uses should be also considered. Such uses comprise human (recreational sports, observing the landscape, walking, camping, and photography, among others) and environmental needs (conservation of aquatic life as haven for wildlife, nature reserves, to mention a few). It should be noted that water scarcity, in terms of both quantity and quality, is also reflected in the ecological resources. These resources are affected in terms of contamination, disconnection, i.e. fluvial systems that no longer reach the sea, shrinkage of lakes, and groundwater depletion. These are the most obvious symptoms of excessive water consumption and poor respect for the ecological systems' recharge rates. In others words it can be attributed to a variety of unsustainable practices.

In addition, climatic change and desertification are thought to play a significant role. In this sense, Hansen et al. (2006) discusses "*exogenous climatic disturbances due to human activities, especially those caused by greenhouse gases, warm the Earth's surface at a rate of about two watts per square meter. Oceans retard the warming effect because they absorb much heat, but it has been found that the global ocean heat content increased by about 10 watt-years per square meter in the last half century*". Climate change will cause variations in rainfall, floods, droughts, changes in the food production factors, and it will contribute to the spread of vector-borne diseases. Climate variability is the leading cause of annual fluctuations in food in both developing and developed countries (FAO, 2003), changes that also impact on water uses. Therefore, climate change could be a factor of pressure on water demand, mainly for those regions which are disadvantaged with droughts and reduced rainfall. It is required that water management becomes more flexible and integrates the climate change as one factor to consider.

1.1.3. New water culture

Much of the water crisis is caused by the way water is used (Abu-Madi and Al-Sa'ed, 2009). In the past the main objectives of water policies were to support the growth of economic aspects and aimed at increasing the water availability to meet with future demands. However, these policies have been excluded from considerations of basic human needs, ecological requirements of water, the roles of community and culture, and the desires and needs of future generations (Gleick, 1998). The benefits of such water policies should be considered against, environmental costs, social and economic disruption.

We live in a transitional stage, which questions the current development model. The conceptualization of science and technology as levers over the "domination" of nature, to promote development based on unlimited growth in a limited world, is being transformed into a more mature approach/paradigm, which seeks to understand the complexity of the environment and to integrate our development in a sustainability perspective[3].

The need for the inclusion of sustainability in water policy began to appear in 1972 at the Stockholm Conference (United Nations, 1972), later in 1977 at the Conference on Water in

[1] http://www.fao.org/nr/water/aquastat/infosystems/indexesp.stm. Last access 07/05/2013.
[2] http://www.unfpa.org/swp/1999/spanish/pdf/resumen.pdf Last access 07/03/2013.
[3] http://www.unizar.es/fnca/index3.php?id=1&pag=11. Last access 08/10/2013.

Mar del Plata (United Nations, 1977), in Dublin in 1992[4], and in the chapter 18 of the Agenda 21 written in Rio[5] among other international reports.

The birth of the concept of sustainable development can be associated to different important findings that human kind began to realize toward the 1970's during the twentieth century: the increase in growth and imbalances, and the existence of limits and environmental impacts (Xercavins et al., 2005).

The Brundtland report (1987) showed the interrelationship between development and environment, and it defined sustainable development as the "*development that meets the needs of present generations without compromising the ability of future generations to meet their own needs*" (Brundtland, 1987).

Sustainability is a multidisciplinary concept, it involves three major blocks that are: society, economy, and environment. Sustainable development means managing and conserving the natural resource basis, and aim institutional and technological changes so as to ensure the continued ability of satisfaction of the needs of present and future generations (Xercavins et al, 2005).

The unsustainable use of water can arise for two reasons:

• Alterations in water stocks and flows, which change its availability in space and time.

• Alterations in water demand caused by changes in living standards, technology, and population levels, among others.

Water availability is affected by both natural and anthropogenic factors, including climate variability, pollution, overexploitation of water sources such as groundwater wells, and other technological factors. Likewise, water demand does not remain constant; it increases along with increases in population, varies with changes in consumer preferences and social values, and could be subject to increments or decrements with technological innovation (Gleick, 1998).

In the context of the current increasing uncertainty of water availability, the need to illuminate perspectives of sustainability from a new development model is bringing a new vast social movement in Spain called the "New Water Culture". The fundamental key of this movement is to highlight the need for a new interdisciplinary approach which, beyond ensuring a fair (reasonable), equitable and efficient use of water as a resource, has to ensure a sustainable management of rivers and aquatic ecosystems. Water culture is, in some way, to understand the complexity of ecosystems.

Arguably, the new water culture has three fundamental principles which are[6]:

• Conservation: understood not only as the attention of the physical and chemical quality of water, but its quality from an ecosystem perspective. Preserve the functionality of rivers, riverbanks and wetlands, means providing prospects of sustainability to the environmental values and services that they provide, such as the renewed availability of quality water resources.

• Efficiency: implies a shift from traditional offer supply strategies to a demand water resources management.

• Territorial Planning: involves integration of water management in the territory with prospects for sustainability.

To move towards this new culture of water radical changes in our values, our conception of nature, our ethical principles, and in our lifestyles are required, that is, there is a need for cultural change. This new culture must follow a holistic approach and recognize the multiple dimensions of ethical, environmental, social, economic, political, emotional values integrated into aquatic ecosystems. And, consequently, they should be managed by communities and

[4] http://www.wmo.int/pages/prog/hwrp/documents/english/icwedece.html. Last access 08/10/2013.
[5] sustainabledevelopment.un.org/content/documents/Agenda21.pdf. Last access 08/10/2013.
[6] http://www.unizar.es/fnca/. Last access 08/10/2013.

public institutions to guarantee an equitable and sustainable management (Vilches et al., 2006).

1.2. Needs for research

The water resources outlook is not promising. Water is a limited resource, today scarce in many world regions in terms of quality and quantity; and it is required for various uses, so competition for its usage across sectors is increasing. This implies the need of integrated water management, taking into account the multiplicity of utilities and functions that water provides, considering that the roots of the water crisis are caused by unsustainable management of water resources, and acting accordingly.

The United Nations has designated the period from 2000 to 2015 as the International Decade for Action "Water for Life", in order to achieve the Millennium Goals, which involve halving of the number of people without access to safe drinking water by 2015 and halt the unsustainable exploitation of water sources[7].

The leaders at all levels, businesses and industries, health and environmental associations, and research or education institutes among others, should focus and work towards solving the current water problems. While better management of water contributes to improving the efficiency of use and conservation of water resources, a moderation in demand and increments on its availability are also of vital importance. It is this last point where improvements in water purification science and technology play a major role (Shannon et al, 2008).

The water challenge faced by providers, managers and scientist is driving exploration into alternative sources of water other than dams and rivers (Stratton and Mattews, 2009). Wastewater reclamation and reclaimed water reuse allow for increasing the water capital without depleting the natural water resources. Thus, treated wastewater reuse has become an essential element of future water resources development in integrated water resources management (Asano, 2002).

The inclusion of planned water reclamation, recycling and reuse in water resource systems reflects the increasing scarcity of water sources to meet societal demands, technological advancements, increased public acceptance, and improved understanding of public health risks. As the link between wastewater, reclaimed water and water reuse has become better understood, increasingly smaller recycle loops are possible (Asano, 2002). The reclaimed water can be used in various applications where quality permits it, thus reducing the demand for potable (drinkable) water, and providing more time for natural sources regeneration. In addition, effluents are kept out of the flow of surface water and groundwater preventing their quality to be harmed, and consequently reducing environmental degradation.

Although wastewater reclamation and reuse is practiced in many countries around the world, the use of reclaimed water is still very low if the total volume of municipal and industrial effluent generated is taken into account (Miller, 2006). Worldwide, more than 368 Km^3 of wastewater are collected annually, from which only 160 Km^3 are treated before rejection into the natural environment, and 7.1 Km^3 are reused (Barceló and Petrovic, 2011). In Spain the practice of reclaiming wastewater is a growing industry. During the last years, about 10 to 13% of the total volume of reclaimed water was reused by year (EPSAR, 2012; Ortega de Miguel and Iglesias Esteban, 2007). A similar situation is observed in Catalonia, where in the year 2008, according to the Agència Catalana de l'Aigua (Catalonian Water Agency)[8] the proportion of reclaimed water used and generated amounted to only 7%, suggesting that the potential for reclaimed water use is enormous.

---

[7] www.un.org/waterforlife/. United Nations. Internacional Decade for action: water for life, 2005-2015, last access 08/05/2013.

[8] http://aca-web.gencat.cat/ Last access 08/12/2013.

Considering worldwide estimates, the industry requires 20% of the global demand of water and agriculture 70%. These sectors can be considered as potential end-users of reclaimed wastewater, since those uses do not demand always drinking water quality and represent the highest percentages of water use. Within the industrial uses, refrigeration is the activity that consumes the highest amount of water, so it is interesting to evaluate the possibility of reusing reclaimed wastewater in these systems, particularly in water cooling towers. However, it is essential that the development of water reuse in agriculture or other sectors is based on scientific evidence about its effects on the environment and public health.

Water reuse is built upon three foundational principles (Asano, 2002):

1. providing reliable treatment of wastewater to meet strict water quality requirements for the intended reuse application,
2. protecting public health; and
3. gaining public acceptance.

Despite large advances in wastewater treatment, waterborne diseases still pose a major world-wide threat to public health (Toze, 1999). Furthermore, new waterborne pathogens are continuously emerging due to changing population demographics, globalization of world trade and travel, and the application of new detection technologies (Nwachcuku and Gerba, 2004). Therefore, one of the key factors of success of reclaimed water use is guaranteeing its microbiological safety. In order to achieve that, the biggest challenge is to remove or inactivate microbial pathogens; and this must be done on a continuous basis. Moreover water utilities stations must be able to convince the public that the recycled water is microbiologically safe for the intended application (Miller, 2006). Consequently, the use of reclaimed municipal water usually requires more stringent monitoring procedures than when good-quality water is used.

To control the associated health risk in reclaimed water use, it is necessary to monitor reclaimed water for various types of microbial pathogens because they have different resistance to treatment and also different infective doses. So, the ability to directly detect pathogens using the most accurate techniques is critical for water managers and providers to confidently assess and manage the risk of existing and new water resources.

Conventional detection methods for pathogen organisms in reclaimed water either rely on culturing them using an artificial medium or cell culture, or, when they cannot be cultured, through direct detection involving the use of microscopy (Toze, 1999). These methods have a variety of serious drawbacks associated with the time taken to isolate and/or identify the pathogen. Sometimes few days are necessary to indicate negative results and up to 7 days for a confirmed positive result. In addition, sometimes high degree of technical skill is required in their application (Bartie et al., 2001) and in many cases the majority of the bacterial population cannot be distinguished from one another under the microscope (Gilbride et al. 2006). Also, the current standard culture methods are unable to detect non-growing bacteria and, thus might not be sufficient for precise monitoring of the microbiological quality of reclaimed water. Current water reclaimed guidelines and recommendations from the World Health Organization (World Health Organization, 1989), Spanish legislation (Royal Decree 1620/2007), and various other state government agencies have based the microbial risk assessment on different counts of indicator organisms such as total coliforms, fecal coliforms, and *Escherichia coli,* and parasite such as helminthes eggs. However, the use of indicator organisms to assess public health risks can have serious limitations, like no correlation to many waterborne pathogens and no valid identification of the pathogen (Szewzyk et al., 2000). Furthermore and more important, these assessments

have not always protected public health to the desirable levels (Jin et al., 2004; Tallon et al., 2005).

To overcome the important drawbacks abovementioned, several alternative and faster methods have been developed. Molecular methods targeting nucleic acids have revolutionized microbial detection, and the use of the polymerase chain reaction (PCR) has proven to be one of the most promising new methods. DNA-based methods, such as real-

time or quantitative PCR (qPCR), are rapid, versatile, sensitive, precise, and allow specific detection and/or quantification of microorganisms of interest in environmental samples. However, apart from inhibition of DNA amplification by substances naturally found in many environmental samples, the inability to differentiate between live and dead cells and the resulting overestimation of microbial targets is considered a major disadvantage of PCR (Wang and Levin, 2006). Whereas the first limitation is greatly remedied by the incorporation of internal amplification controls, the second one is being addressed by pretreating the sample with viability dyes, such as ethidium monoazide (EMA) and propidium monoazide (PMA) before DNA extraction and amplification (Nocker et al., 2006; Nogva et al., 2003). Viable PCR is a promising technique because it makes use of the speed and sensitivity of the molecular detection providing at the same time viability information. In the last years, it has evolved into a commonly applied method. The addition of a pretreatment step to the sample analysis to inhibit the amplification of DNA from damaged membrane cells has been used in combination with different molecular techniques (Brescia et al. 2009; Graiver et al. 2010; Lu et al. 2009; Nocker et al. 2007; Nocker et al. 2009; Nocker et al. 2010; Rogers et al. 2008; Rudi et al. 2005). Despite providing evidence of feasibility, the research has illustrated the urgent need for adding viability information to DNA-based diagnostics in diverse fields ranging from testing of food and water safety to clinical microbiology.

Research is needed to reduce persistent uncertainty about the potential adverse effects that may have use of reclaimed water on human health and the environment, with the ultimate goal of increasing confidence in reuse practices and public acceptance. Improved detection of microbial pathogens in reclaimed water will be essential to help optimizing all steps in treatment and use, and thus to encourage the use of reclaimed wastewater in agricultural and industrial uses. PCR has the potential to be one of the quickest and useful methods available for microbial pathogen detection. It can be a useful tool for water quality monitoring and control.

1.3. Objectives and overview of the dissertation

In order to contribute to the sustainable and safe-use of reclaimed water, the main objectives of this work have been to increase the available knowledge on microbiological quality of reclaimed water and its monitoring, and on the potential sanitary risk associated to agricultural and industrial reclaimed water use practices.

To achieve these main objectives, work was performed along two main intertwined research lines: (1) the development, validation and improvement of qPCR methods to detect and quantify waterborne microorganisms in water, in order to get a fast and effective tool for controlling and monitoring the reclaimed water microbiological quality; (2) the study of microbiological colonization associated to reclaimed water use at pilot scale practices in the Catalonian region using culture and qPCR techniques.

Different specific objectives were accomplished through the different dissertation chapters. The motivation and importance of studying reclaimed water scenarios as well as the improvement in microbiological quality control tools are briefly described in Chapter 1. Chapter 2 summarizes relevant concepts about wastewater and reclaimed water, the health aspect related to water reuse, and the methodologies used for monitoring microorganisms in water.

Regarding the first work line, an initial approximation to molecular methods was performed. Chapter 3 focuses in the development and validation of a qPCR method to detect *Legionella pneumophila* in cooling water samples. *Legionella* bacterium was studied because it represents an important health concern in water systems, especially in those in which water aerosolization happen, such as cooling towers which, as aforementioned, are main water users in the industry and potential reclaimed water consumers. A deep work was performed with viability qPCR aiming at improving this technique, especially to be used to analyze

environmental samples. The viability qPCR technique was validated using cultured and environmental samples. Chapter 5 and 6 depicted some studies of PMA pretreatment application to detect and quantify viable microorganisms using molecular methods. In Chapter 5 the ability of viability qPCR to quantify live *Legionella pneumophila* in cultured samples in the presence of dead cells has been evaluated. Some problems to quantify properly viable cells by PMA-qPCR in the presence of high levels of non-viable cells were observed. For that reason, a strategy for overcoming this and other problems associated to the use and application of the viability qPCR technique was proposed and analyzed in Chapter 6. This strategy was based on the combination of three qPCR amplifications for each sample to provide an improved estimation of the number of live cells.

About the second work line, two different pilot systems were constructed and studied to evaluate different reclaimed water uses, such as industrial and agricultural applications. In the case of industrial water reuse, the analysis of cooling water systems was undertaken. The specific objectives in this study, depicted in the Chapter 4, were the verification of the effect of water origin in the *Legionella* colonization of cooling towers demonstrative units by qPCR; and the microbiological colonization monitoring of a cooling tower pilot systems when *in situ* disinfection was performed by using qPCR. In some studies viability qPCR has also been applied.

Another pilot study, depicted in Chapter 7 was encompassed to ascertain the safety of irrigating vegetables crops with reclaimed water. Four different water sources were used and analyzed. Extensive microbiological analysis of water and vegetable tissue samples were conducted by culture and viability qPCR methods during the field studies.

Some final discussion and conclusions related to the performed work are summarized and presented in Chapter 8.

1.4. References

Abu-Madi, M., Al-Sa'ed, R. 2009. Towards sustainable wastewater reuse in the middle East and North Africa. J. Sustain. Dev., 2, pp. 1475-1481.

Asano, T. 2002. Water from (waste) water- The dependable water resource. Water Sci. Technol., 45, pp. 24-33.

Barceló, D., Petrovic, M. 2011. Waste water treatment and reuse in the Mediterranean rRegion. Springer Verlag GmbH, Berlin, Germany.

Bartie, C., Venter, S.N., Nel, L.H. 2001. Evaluation of detection methods for *Legionella* species using seeded water samples. Water SA, 27, pp. 523-527.

Bixio, D., Thoeye, C., De Koning, J., Joksimovic, D., Savic, D., Wintgens, T., Melin, T. 2006. Wastewater reuse in Europe. Desalination, 187, pp. 89-101.

Bouwer, H. 2002. Integrated water management for the 21st century: problems and solutions. J. Irrig. Drain Eng., 128, pp. 193-202.

Brescia, C.C., Griffin, S.M., Ware, M.W., Varughese, E.A., Egorov, A.I., Villegas, E.N. 2009. *Cryptosporidium* propidium monoazide-PCR, a molecular biology-based technique for genotyping of viable *Cryptosporidium* oocysts. Appl. Environ. Microbiol., 75, pp. 6856-6863.

Brundtland, J. 1987. Brundtland inform. World Commission on the United Nations. Conference on Environment and Development. New York, US.

Engelman, R., Cincotta, R.P., Dye, B., Gardner-Outlaw, T., Wisnewski, J. 2000. People in balance. Population Action International, Washington, DC, US.

EPSAR (Entidad Pública de Saneamiento de Aguas Residuales de la Comunidad Valenciana). 2012. Reutilización de aguas depuradas en la comunidad valenciana. Oral presentation. IX Congreso Internacional AEDyR. November, 13-15. Madrid, Spain.

FAO. 2003. World agriculture: Toward 2015/2030, Chapter 13. Rome, Earthscan.

Gernjak, W. 2006. Solar photo-fenton treatment of priority substances-process parameters and control strategies. Doctoral thesis dissertation. University of Natural Resources and Applied Life Science, Vienna.

Gilbride, K.A., Lee, D.Y., Beaudette, L.A. 2006. Molecular techniques in wastewater: understanding microbial communities, detecting pathogens, and real time process control. J. Microbiol. Methods, 66, pp. 1-20.

Gleick, P.H. 1998. Water in crisis: paths to sustainable water use. Ecol. Appl., 8, pp. 571–579.

Graiver, D.A., Saunders, S.E., Topliff, C.L., Kelling, C.L., Bartelt-Hunt, S.L. 2010. Ethidium monoazide does not inhibit RT-PCR amplification of nonviable avian influenza RNA. J. Virol. Methods, 164, pp. 51-54.

Hansen, J., Sato, M., Ruedy, R., Lo, K., Lea, D.W., Medina-Elizade, M. 2006. Global temperature change. PNAS, 103, pp. 14288-14293.

Hochstrat, R., Wintgens, T. (Eds.). 2003. AQUAREC, report on milestone M3.I, draft wastewater reuse potential estimation, interim report.

Jin, G., Jeng, H.W., Bradford, H., Englande, A.J. 2004. Comparison of *E. coli*, enterococci, and fecal coliforms as indicators for brackish water quality assessment. Water Environ. Res., 76, pp. 245-255.

Liu, L., Johnson, H.L., Cousens, S., Perin, J., Scott, S., Lawn, J.E., Rudan, I., Campbell, H., Cibulskis, R., Li, M., Mathers, C., Black, R.E. 2012. Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. Lancet, 379(9832), pp. 2151-2161.

Loutfy, N.M. 2011. Reuse of wastewater in Mediterranean region, Egyptian experience. In: Barceló, D., Petrovic, M. (Eds.). 2011. Waste water treatment and reuse in the Mediterranean region. Springer, Berlin, Germany, pp. 183-213.

Lu, Y., Yang, W., Shi, L., Li, L., Alam, M.J., Guo, S., Miyoshi, S. 2009. Specific detection of viable *Salmonella* cells by an ethidium-monoazide-loop mediated isothermal amplification (EMA-LAMP) method. J. Health Sci., 55, pp. 820-824.

Miller, G.W. 2006. Integrated concepts in water reuse: managing global water needs. Desalination, 187, pp. 65-75.

Nocker, A., Cheung, C.Y., Camper, A.K. 2006. Comparison of propidium monoazide and ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Methods*,* 67, pp. 310–320.

Nocker, A., Sossa-Fernandez, P., Burr, M.D., Camper, A.K. 2007. Use of propidium monoazide for live/dead distinction in microbial ecology. Appl. Environ. Microbiol., 73, pp. 5111–5117.

Nocker, A., Mazza, A., Masson, L., Camper, A.K., Brousseau, R. 2009. Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology. J. Microbiol. Methods, 76, pp. 253–261.

Nocker, A., Richter, T., Schuren, F., Montijn, R., Kort, R. 2010. Live/dead discrimination in 454 pyrosequencing based bacterial community analysis of environmental water samples. Int. Microbiol., 13, pp. 59-65.

Nogva, H.K., Dromtorp, S.M., Nissen, H., Rudi, K. 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5′-nuclease PCR. Biotechniques, 810, pp. 812–813.

Nwachcuku, N., Gerba, C.P. 2004. Emerging waterborne pathogens: can we kill them all?. Curr. Opin. Biotechnol., 15, pp.175-180.

Organisation for Economic Cooperation and Development (OECD). 2003. Environmental performance reviews: water – performance and challenges in the OECD countries. In Bixio, D., Thoeye, C., De Koning, J., Joksimovic, D., Savic, D., Wintgens, T., Melin, T. 2006. Wastewater reuse in Europe. Desalination, 187, pp. 89-101.

Ortega de Miguel, E., Iglesias Esteban, R. 2007. La reutilización de las aguas depuradas en España. Normativa y tecnologías más utilizadas. SMALLWAT, Sevilla, España.

UNDP (United Nations Development Programme). 2006. Human development report 2006: beyond scarcity: power, poverty and the global water crisis. Palgrave Macmillan, New York, US.

Rogers, G.B., Stressmann, F.A., Koller, G., Daniels, T., Carroll, M.P., Bruce, K.D. 2008. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. Diagn. Microbiol. Infect. Dis., 62, pp. 133-141.

Royal Decree-Law 1620/2007 of treated wastewater reuse. 2007. Official Bulletin of State, BOE, 294.

Rudi, K., Moen, B., Drømtorp, S.M., Holck, L. 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl. Environ. Microbiol. 71, pp. 1018–1024.

Shannon, M.A., Bohn, P.W., Elimelech, M., Georgiadis, J.G., Mariñas, B.J., Mayes, A.M. 2008. Science and technology for water purification in the coming decades. Nature, 452, pp. 301-310.

Stratton, H., Matthews, B. 2009. Pathogens in recycled water: are they measurable?. Microbiol. Australia, 30, pp. 12-15.

Szewzyk, U., Szewzyk, R., Manz. W., Schleifer, K.H. 2000. Microbiological safety of drinking water. Annu. Rev. Microbiol., 54, pp. 81-127.

Tallon, P., Magajma, B., Lofranco, C., Leung, K.T. 2005. Microbial indicators of faecal contamination in water: a current perspective. Water, Air, Soil Poll., 166, pp. 139-166.

Toze, S. 1999. PCR and the detection of microbiological pathogens in water and wastewater. Wat. Res., 33, pp. 3545-3556.

United Nations. 1972. Report of the United Nations cConference on the human environment. U.N. Doc. A/CONF.48/14, at 2-65. Stockholm, Sweden. http://sustainabledevelopment.un.org/index.php?menu=1373

United Nations. 1977. Report of the United Nations water conference. United Nation Publication, Sales No.E. 77. II.A.12. Mar del Plata, Argentina.

Vilches, A., Gil Pérez, D., Toscano, J.C., Macías, O. 2006. Nueva cultura del agua. OEI. http://www.oei.es/decada/accion06.htm

Wang, S., Levin, R.E. 2006. Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. J. Microbiol. Methods, 64, pp. 1–8.

World Health Organization (WHO). 1989. Health guidelines for the use of wastewater in agriculture and aquaculture. Report of a WHO Scientific Group. Technical Report Series, No. 778. WHO, Geneva, Switzerland.

World Health Organization (WHO) and UNICEF. 2005. Water for life: making it happen. WHO Press, Geneva, Switzerland.

World Health Organization (WHO) and UNICEF. 2012. Progress on Drinking Water and Sanitation: 2012 Update. WHO Library Cataloguing-in-Publication Data. US.

Xercavins, J., Cayuela, D., Cervantes, G., Sabater, A. 2005. Desarrollo sostenible. Edicions UPC. Barcelona, España.

Chapter 2

Background

2.1. Water reclamation

2.1.1. Definitions and historical development

Wastewater can be defined as used water discharged from homes, business, cities, industry, and agriculture (Tchobanoglous et al., 2003). It is water that has been adversely affected in quality by anthropogenic influence, so the generation of wastewater has uninterruptedly enlarged over the years as the human population has increased.

The treatment process required to make wastewater reusable in a beneficial manner is usually called regeneration or reclamation, and the outcome of this process is herein called reclaimed water. According to its etymological meaning, the reclamation of water returns, partially or totally, the quality level it had before being used (Mujeriego, 2006).

The origin of the water reuse goes back thousands of years. According to Asano and Levine (1996) it began around 3000 BC, and from that time up to the present, three stages can be identified: initial period (3000 BC - 1850), sanitary awakening period (1850 - 1950), and reuse period (1950 - present).

- *Initial Period (3000 BC - 1850)*: In the Mediterranean basin, wastewater recycling and reuse were practiced since the Ancient Greek and Roman civilizations (Angelakis and Spyridakis, 1996). The first wastewater reuse for agricultural irrigation corresponds to 3000 BC and belonged to the Minoan Civilization in ancient Greece. Around 97 AD, there is evidence of the existence of a water supply commissioner, Sextus Julius Frontius, in the city of Rome. Sewage farm practices have been recorded in Germany and United Kingdom since XVI and XVIII centuries respectively (Vigneswaron and Sundaravadivel, 2004). From 1800, the legal use of sewers for human waste disposal in cities like London, Paris and Boston, was instituted. After

the cholera epidemic in London between 1848 and 1854, a sanitary code was published in Great Britain which stated "the rain to the river and the sewage to the soil."

- *Sanitary Awakening (1850 - 1950)*: during this period water supply was linked to diseases that resulted in important advances in health and engineering fields. Some important events during this period were the control of cholera epidemic in London by John Snow in 1850, the development of the theory of typhoid fever prevention by Bud in England, the advances in microbiology in Germany and France by Koch and Pasteur, respectively, the use of chlorine as a disinfectant, the knowledge of the disinfection kinetic (Chick law), and the use of biological processes to treat wastewater by Ardem and Lockett in England in 1904.
- *Reuse and recycling of water (1950 - present)*: The planned reuse of water began in the early 20's in the United States (US), specifically in the states of Arizona and California, using the reclaimed water for agricultural purposes. In Colorado and Florida systems were developed for reuse in urban uses. The rules for the reuse began in California at the same time (1918). Since 1965, these rules have played a crucial role in the regeneration, recycling, and reuse of wastewater. Growing population, higher water demand, and technological advances in physical, chemical, and biological processing of wastewater led to the contemporary era of water reclamation and reuse (Metcalf & Eddy, 2007).

2.1.2. Wastewater treatment

The wastewater treatment is a combination of operations including physical, chemical, and biological processes, which is used to remove or reduce the contaminants found in wastewater like biodegradable organic compounds, volatile organic compound, recalcitrant xenobiotics, toxic metals, suspended solids, nutrients (nitrogen and phosphorus), and microbial pathogens and parasites (Bitton, 2005). It takes place in wastewater treatment plants (WWTP).

Briefly, the wastewater treatment comprises the following steps:

• Pretreatment: the objective of this operation is to remove large debris and coarse materials that could cause damage or/and clogging problems in the processing (treatment facility’s) equipment. Preliminary treatment operations include (Stenco, 2007):

- Screening: removal of the big solids (rags, stricks, plastic, wood, etc.) to protect pumps and prevent clogs in pipes and valves. These solids are disposed directly in a landfill site, or instead they are subjected to a treatment such as incineration.
- Grit removal: separation of gravel, sand, and mineral particles in suspension to avoid sediment deposition in canals and pipes, and also to prevent overloading of the final sludge. The separation is done by gravity using a grit chamber.
- Oils and fats separation: it is performed to avoid problems in the subsequent processes due to the presence of fats that could make difficult the suspended solid separation, and prevent proper ventilation.
- Homogenization: it allows feed continuously the treatment plant with a consistent quality effluent.

• Primary treatment: it consists in a physico-chemical purification which aims are:

- Remove the settleable organic and inorganic matter. Settling can be improved by adding coagulants and flocculants. The primary decantation can remove approximately 25 to 50% of biochemical oxygen demand (BOD), between 50 and 70% of suspended solids, and 65% of oil and grease (Mujeriego, 1990).
- Remove floating matter and foam from the liquid surface by means of a skimming device.

The operation is performed in large tanks named primary clarifiers. The settled solids, usually called primary sludge, are pumped along with floating material to anaerobic digesters for further treatment.

• Secondary treatment: it consists in a biological treatment which involves the removal of biodegradable organic matter, both dissolved and colloidal, by an aerobic and/or anaerobic biological process. In this process the organic matter is metabolized by a biomass of microorganisms. Once the organic matter is assimilated by the microorganisms, the effluent is transferred to secondary clarifiers where the biological solids or sludges are settled by gravity, in some cases it also can be done by flotation (Metcalf & Eddy, 1991). Nutrients removal also generally occurs in this wastewater treatment stage.

• Tertiary treatment: an additional treatment is performed to remove remaining suspended and dissolved substances, and thus to achieve a higher quality effluent than that obtained from the conventional secondary treatment. This may be accomplished by using physical, chemical, or biological treatment processes, and it generally consists of a filtration (using double-layer filters, activated carbon or membranes), and a disinfection. Bacteria, viruses, and parasites, which are harmful to public health, are removed at this stage. It is an essential step in wastewater treatment systems and wastewater reuse because it allows minimize the direct and indirect risks of reclaimed water use for: the environment, the people will use it, the populations surrounding the user areas, and the consumer of products whose production process uses reclaimed water.

2.2. Health aspects of reclaimed water use

Reclaimed water is a complex resource, with both advantages and inconveniences for its uses. When used, it is essential to consider the possible adverse effects on the environment and on public health in order to minimize or avoid the impacts on them.

It is important to note that health hazards are one of the main constraints for treated wastewater reuse (Salgot et al., 2006). There are two categories of health effects related to reclaimed water use: the ones due to biological agents, and those due to the chemical agents. The first ones have been recognized since the very beginning of water reuse; they pose the greatest or most recognized health risks given that are linked to relatively immediate outcome (short-term risk). They are, therefore, subject to strict limitations by quality standards. The health risks related to chemical agents have a relatively time-delayed outcome (long-term risk) and have been related to water reuse following improvements in analytical capabilities (Lazarova et al., 2005).

2.2.1. Chemical agents

Industrial, agricultural, and domestic uses add chemical constituents to water. The chemical agents present in wastewater can be broadly classified into organic and inorganic compounds. Dissolved constituents, nutrients, nonmetallic constituents, metal, and gases can be found as inorganic compounds. Some organic contaminants found in wastewater can be humic substances, high molecular weight aliphatic and aromatic hydrocarbons, fats, oils, synthetic organic chemicals, and microorganism’s metabolites. Humic material may serve as precursors in the formation of disinfection byproducts, such as trihalomethane, during the disinfection process (Metcalf & Eddy, 2007). Also the presence of various emerging contaminants like pharmaceutically-active compound (e.g. analgesics, antibiotics, and antidepressants), endocrine disrupting compounds (e.g. estradiol, phytoestrogens, pesticides, industrial chemicals such as bisphenol A, and nonyl phenol), and hormones in wastewater and reclaimed water has become a concern in the last decades (Bolong et al.,

2009; Kümmerer, 2009; Sui et al., 2010; Yang et al., 2011). They are potential carcinogens, mutagenic, and long-term toxics (Fenton, 2006; Metcalf & Eddy, 2007).

The need to control chemical contaminants arises when the reclaimed water is aimed at uses where public exposure is more direct and the potential for ingestion (accidental or not) is higher, such as potable direct use, and irrigation. The concern is related to the possible contaminants accumulation in the environments, and their possible incorporation into the food chain. The endocrine disrupting compounds can impact on the function and structure of an organism's endocrine system causing effects on the organism or its progeny (Lim et al., 2000). Regarding pharmaceutically-active compounds, some antibiotic resistance can be developed for soil and water microorganisms (Guardabassi et al., 1998). The presence of hormones may induce their endogenous production, which might cause alterations in the aquatic life (Petrovic and Barceló, 2012; González et al., 2012).

Some dissolved inorganic compounds can produce salt accumulation in soil which are associated with adverse effects on both crop (productivity, crop transpiration, and growth) and soil (soil permeability, clay particles) (Lazarova et al., 2005). Groundwater quality can also be impacted by the leaching of chemicals present in contaminated irrigation water (Metcalf & Eddy, 2007). Nutrients in high concentrations can produce eutrophication in aquatic environments, which can stimulate the growth of algae, increased water purification costs, and interference with the recreational value of water (Akpor and Muchie, 2011).

Some studies have shown that conventional wastewater treatment plants can not completely remove many pharmaceutically-active compound and endocrine disrupting compounds. Consequently, advanced tertiary treatment is necessary for their removal (Caliman and Gavrilescu, 2009; Nakada et al., 2006; Sui et al., 2010; Yang et al., 2011).

2.2.2. Biological agents

The main biological agents found in wastewater can be classified into three major groups: bacteria, parasites (such as protozoa and helminths), and viruses. More importantly, most of them are enteric in origin (Toze, 2006). They derive mainly from infected humans and other warm-blooded animals, can survive in water, and are transmitted directly or indirectly by the waterborne route (Bitton, 2005; Metcalf & Eddy, 2007). Pathogenic microorganisms commonly gain access to the host through the gastrointestinal tract, the respiratory tract, and the skin. Table 2.1 shows the microorganisms commonly found in untreated wastewater and the diseases associated with them. It is important to note that the risk of waterborne infection from these microorganisms depend on a range of factors like pathogen number and dispersion in water, the infective dose required and the susceptibility of an exposed population, and the water treatment undertaken before potential exposure to the water (Haas et al., 1999).

The number of pathogenic microorganisms in reclaimed water has decreased considerably in recent decades due to improvements in sanitation and optimization of the technologies used. However, special care must be taken to minimize health risks and ensure the acceptability and safety of the use of reclaimed water for a given reuse application.

Bacteria are distributed ubiquitously in nature and represent the largest group of organisms found in reclaimed water. They are small (0.2 to 10 μm) unicellular prokaryotic organisms, and can be classified by structure (morphology), response to chemical stains, nutrition, and metabolism (Gerardi, 2006). According Metcalf & Eddy (2007) some important members of this group are: *Shigella*, *Salmonella*, *Escherichia coli*, *Yersinia enterolitica*, and *Campylobacter jejuni*. Finding them in reclaimed water represents a high risk of gastrointestinal illness associated with the use of such water. In addition, opportunistic pathogens such as *Pseudomona aeruginosa*, *Legionella*, *Aeromonas*, and *Mycobacterium* can be found in reclaimed water. They are not enteric in origin, because they are common inhabitants of soil and/or water, and may cause disease in susceptible individuals, such as young, elderly, and immunocompromised humans (Bitton, 2005; Jjemba et al., 2010).

Table 2.1. Biological agents potentially present in untreated wastewater.

MICROORGANISMS	ASSOCIATED DISEASE
BACTERIA	
Escherichia coli (enterotoxigénic)	**Gastroenteritis**
Salmonella enterica serovar *Typhi*	**Typhoid**
Salmonella enterica (2500 serotype)	**Salmonellosis (diarrhea)**
Shigella (4 spp)	**Bacillary dysentery**
Yersinia enterocolitica	**Gastroenteritis**
Campylobacter	**Gastroenteritis**
Vibrio cholerae	**Cholera**
Leptospira (spp.)	**Leptospirosis**
Helycobacter pylori	**Peptic ulcers, stomach cancer**
Legionella	**Pneumonia and other respiratory infections**
PROTOZOA	
Balantidium coli	**Balantidiasis (dysentery)**
Cryptosporidium parvum	**Cryptosporidiosis**
Entamoeba histolytica	**Amoebic dysentery**
Entamoeba histolytica	**Amoebic dysentery**
HELMINTHS	
Ascaris lumbricoides	**Ascariasis**
Taenia solium and Taenia saginata	**Taeniasis**
Trichuris trichiura	**Trichuriasis**
VIRUSES	
Enteroviruses (72 types)	**Gastroenteritis, heart anomalies, and meningitis.**
Hepatitis A	**Infectious hepatitis**
Norwalk virus	**Gastroenteritis**
Rotavirus	**Gastroenteritis (infantile)**
Adenovirus (31 types)	**Respiratory disease, eye infection, diarrhea**

Adapted from (Crites and Tchobanoglous, 1998).

Viruses are very small colloidal particles (25-350 nm), and are obligate intracellular parasites (Bitton, 2005). Their infective dose is generally lower than for bacterial pathogens, and the infected cells may be animal or plant cells, bacteria, fungi or algae. Wastewater may become contaminated by approximately 140 types of enteric viruses, such as enteroviruses, rotaviruses, noroviruses, hepatitis A virus, adenoviruses, and reoviruses. They are responsible for a broad spectrum of diseases including skin rash, fever, respiratory infections, conjunctivitis, myocarditis, aseptic meningitis, herpangia, gastroenteritis, and paralysis (Jjemba et al., 2010).

Protozoan parasites are single-celled organisms that typically are larger than bacteria (Metcalf & Eddy, 2007). They are released into aquatic environments as cysts or oocysts which are quite resistant to environmental stress and to disinfection, and do not multiply outside their hosts (Bitton, 2005). Important pathogenic protozoans in wastewater include *Crypstosporidium parvum*, *Giardia liamblia*, and *Entoamoeba histolytica*. Also, amoebae may

play an important role in the public health risk associated with wastewater reuse applications since it has been demonstrated that some pathogenic microorganisms can grow and/or survive in free-living amoebae (Thomas et al., 2010). Amoebae can colonize virtually any kind of water system and support harsh physical and chemical conditions, so the presence of amoebae could indicate possible pathogen survival (Codony et al., 2012).

Helminths are worm-like parasites. They are invertebrates characterized by elongated, flat or round bodies, and develop through egg, larval (juvenile), and adult stages (Castro, 1996), being the egg the infective stage (Bitton, 2005). Egg helminths are very resistant to environmental stresses and usual wastewater disinfection treatment such as chlorination (Jjemba et al., 2010).

In this research work some relevant bacteria were specifically monitored in reclaimed water, as well as their presence in microbial communities, such as biofilms. A short description of them is given below.

Total aerobic bacteria. Total aerobic bacteria include the aerobic and facultative anaerobic bacteria that derive their carbon and energy from organic compounds (Bitton, 2005). Aerobic mesophilic organisms reflect the existence of favorable conditions for the multiplication of microorganisms (Aycicek et al., 2006) and allow assessing the microbial inactivation or removal efficiency in wastewater treatment.

Total coliform bacteria. Coliforms include several Enterobacteriaceae bacteria of which *Escherichia coli* is the most important (Metcalf & Eddy, 2007). They are aerobic and facultative anaerobes Gram-negative rod shaped bacteria. Their presence in water indicates that disease-causing organisms could be in the water and/or in the water system. Fecal coliform bacteria are a sub-group of total coliform bacteria that mostly exist in feces of warm-blooded animals. Their presence in water indicates recent fecal contamination.

Escherichia coli. It belongs to the fecal coliform group. There are several strains of *Escherichia coli*, many of which are harmless. However, some of them are pathogenic and can cause gastroenteritis and serious diarrhea in humans; *Escherichia coli* serotype O157:H7 is one of them. Several outbreaks caused by this pathogen were shown to be associated with waterborne transmission (Keene et al., 1994; Nataro and Kaper, 1998; Word Health Organization, 2008).

Enterococci. These bacteria are natural habitants of human and animal gastrointestinal tract, and some species may cause serious infections such as urinary tract infections, bacteremia, endocarditis, neonatal sepsis, and rarely meningitis (Sood et al., 2008). Enterococci species are Gram-positive facultative anaerobic organisms that can survive and grow in many environments. The reason of their surveillance is related to their resistance to environmental extreme conditions and to a large number of antimicrobial agents (Moellering, 1991). Consequently, they can be used as persistent hygiene indicators in water.

Bacteroides spp. They are anaerobic Gram-negative rods (Wexler, 2007). *Bacteroides* spp. positive detection can be used as an interesting indicator of faecal pollution because of their abundance in the gastrointestinal tract of humans and warm-blooded animals, with host-specific distribution (Savichtcheva and Okabe, 2006). However, it is not normally used for routine analysis because their fastidiousness. They are difficult to isolate by culture methods. In spite of this, with the introduction of the molecular techniques, the gender *Bacteroides* is usually used as an indicator of fecal pollution (Savichtcheva et al., 2007; Savichtcheva and Okabe, 2009).

Helycobacter pylori. They are ubiquitous micro-aerobic Gram-negative bacteria that cause gastritis, peptic ulcer disease, functional dyspepsia, and cancer (Gasparetto et al., 2012). *Helicobacter pylori* have been detected in non-treated water (Nayak and Rose, 2007) and in drinking water (Baker and Hegarty, 2001), which suggested that water might be an important infection source as was suggested by Hulten et al. (1996).

Legionella pneumophila. Legionella are Gram-negative coccobacilli (Diederen, 2008). Legionellaceae family consists of a single genus, *Legionella*, which comprises more than 50 different species and 70 different serogroups. Of all the species, *Legionella pneumophila*

highlights for its greater pathogenicity, accounting for about 90% of cases of Legionnaires' disease (Benin et al., 2002). *Legionella pneumophila* includes at least 16 different serogroups (Benson and Fields, 1998) of which serogroups 1, 4, and 6 are responsible for majority of human infections (Vergis et al., 2000).

Legionnaires' disease (Legionellosis), first described in Philadelphia (US) in 1976, is a type of pneumonia which may also involve the gastrointestinal and urinary tracts, as well as the nervous system. *Legionella* is also the causative agent of the Pontiac fever that is a milder respiratory infection without pneumonia that resembles a severe flu manifested by fever, headaches and muscle aches, but does not require any treatment (Kaufmann et al., 1981).

Legionellosis is a significant health problem in many countries leading to the death of around 500 European citizens every year; therefore effective preventive strategies are needed (Beauté et al., 2013; Carratalà and García Vidal, 2010). It is a notifiable disease in Spain since 1995 (Royal Decree 2210/1995, 1996). According to the data analysis from the European Legionnaires' Disease Surveillance Network, Spain is among the European Union (EU) countries with higher incidence of this disease. The incidence rates were 2.66 and 2.45 cases per million population in 2009 and 2010, respectively, while the European average incidence in 2009 and 2010 stood at 1.07 and 1.2 cases per million population, respectively (Beauté et al., 2013).

Legionella are found in aquatic environment and soil (Bitton, 2005; Diereden, 2008). This pathogen can develop in association with other bacteria such as amoeba or ciliates, resulting in greater resistance to biocides, chlorination, low pH, and high temperatures (Bitton, 2005; Codony et al., 2012; García et al., 2013; Thomas et al., 2004).

Respiratory infections caused by *Legionella pneumophila* are mainly attributed to the inhalation of contaminated aerosols (Stout and Yu, 1997) produced by systems such as cooling towers (Anonymous, 1994), showers (Rosmini et al., 1984) and nebulizers (Mastro et al., 1991). The aspiration has also been proposed as a possible transmission mechanism (Blatt et al., 1993; Yu, 1993).

Facilities that are most often contaminated with *Legionella* and have been identified as major sources of infection are domestic water systems, cooling towers, and evaporative condensers. Others, less important, are ornamental fountains, irrigation systems, humidifiers and hydrotherapy facilities. Among the former, cooling towers are one of the main causes of the most important outbreaks. It is worth mentioning that in Spain, from 1999 to 2009, there have been 501 outbreaks of Legionellosis. The source of infection was unknown in the 55.5% of the reported outbreaks (278). Regarding the outbreaks in which the possible source was determined (confirmed or suspected), domestic water system were involved in 110 (49.3%) of them, refrigeration towers or other similar devices such as evaporative condensers were the source in 90 (40.3%) of them, bathrooms with water movement were implicated in 10 (4.5%) of them, and other mechanisms (e.g. humidifiers, an open tank, a ship water installation, etc.) were involved in 12 (5.4%) of them (Cano Portero et al., 2010).

Circulation water systems, including cooling towers, offer the ideal conditions for incubation and proliferation of microorganisms. These systems provide with adequate oxygen saturation, sunlight exposure, temperatures between 30 and 60 °C, and pH values between 6 and 9. All these factors are combined with the existence of enough nutrients for maintaining microbial life. Furthermore, biofilms and sediments found in the recirculating water systems are a potential niche for the growth of *Legionella* and other bacteria (Declerck, 2010; Green and Pirrie, 1993; Kooij and Veenendaal, 2002; Koubar et al., 2013). Biofilms are directly related to recontamination problems in water systems (Johansen et al., 1997); they favor the resistance to antimicrobial compounds (Green and Pirrie, 1993; Kool, 2002), reduce heat transfer in thermal systems (Wright et al., 1991), and cause corrosion (Momba and Binda, 2002).

Biofilms are complex communities of microorganisms enclosed in an extracellular polymeric substance attached to a surface, which may consist of only one species of microorganisms, but generally have a range of them (Costerton, 1995). The biofilm begins to form when a single cell adheres to a surface. The ability of the cell to carry out this distinct growth phase

depends on environmental factors such as temperature and pH, and genetic factors that code for motor functions, environmental sensitivity, adhesins and other proteins (Costerton, 1995; O'Toole et al., 2000). After initial binding, the cell begins to grow and spread on the surface in a monolayer while forming micro-colonies. The cells change their behavior leading to the formation of a complex structure. The microorganisms form an expolysaccharide matrix that embeds the biofilm structure (Danese et al., 2000; Flemming and Wingender, 2010). As the biofilm grows other changes occur. If conditions are favorable, it may spread to clean surfaces or release some cells, which retrieves the qualities of planktonic cells.

Aggregate microorganisms exhibit properties, behavior, and survival strategies that far exceed their capabilities as individual bacteria. The exopolymeric matrix protects microorganisms from the nutrient dispersion, desiccation, and makes them highly resistant to antimicrobial agents (Mah and O'Toole, 2001; Fux et al., 2005).

Biofilms are ubiquitous (Wingender and Flemming, 2011). Therefore they can be found in aquatic and industrial water systems serving as a reservoir for pathogenic microorganisms and representing a potential source of water contamination, resulting in a potential health risk for humans. Several types of microorganisms were found within biofilms. The most alarming results are the presence of pathogens in them such as *Pseudomonas*, *Mycobacterium*, *Campylobacter*, *Klebsiella*, *Aeromonas*, *Legionella* spp., *Helicobacter pylori,* and *Salmonella enterica* serovar *Typhimurium* (Armon et al., 1997; Burke et al., 1984; Engel et al., 1980; Mackey et al., 1998).

Biofilms are important in water reclamation and reclaimed water use. They play an important "good" role in the wastewater treatment by biodegrading organic contaminants as part of the secondary water treatment (Bishop, 2007). However, they must be controlled due to biofouling problems or the growth of pathogenic or other unwanted microbes within their matrix.

With proper attention to water quality standards, a water reclamation plant can reliably produce high-quality water which should not pose an increased risk of disease to those using the water. To that end, proper surveillance and monitoring of water quality for wastewater reuse should be performed frequently.

2.3. Reclaimed water uses

2.3.1. Type of reclaimed water uses

Wastewater regeneration provides water to serve a variety of beneficial uses, leaving more freshwater for the environment, and reducing the volume of wastewater discharged to receiving water bodies.

Taking into consideration the possible contact or ingestion of reclaimed water by the people, water reuse is classified as:

• potable use: it refers to the use of highly quality reclaimed water to augment drinking water supplies (Rogers et al., 2006)

- indirect potable use: reclaimed water is mixed with a natural water body, such as surface or groundwater, prior to drinking water treatment.
- direct potable use: reclaimed water is introduced directly into the drinking water distribution system. Its use is limited to extreme cases (Asano, 1998).

• non-potable reuse: it includes water reuse applications different than potable water reuse, and it constitutes a large majority of reclamation water use in the world (Rogers et al., 2006). It also can be direct or indirect depending if reclaimed water pass or not through a natural water body before its use.Regarding non-potable use, reclaimed water has been used for a variety of purposes like:

- urban uses (gardening, fire, street, and car washing service),
- industrial uses (cooling, washing of railway carriages, material transport),
- agricultural and forest irrigation,
- ornamental and recreational uses (ornamental fountains, lakes, ponds, irrigation of golf courses),
- improvement and preservation of the environment.

It is important to note that wastewater regeneration projects for non-potable uses are those who have acquired the further development in many parts of the world. They have achieved excellent levels of reliability and acceptance by users and the public general, especially in developed countries where water resources are limited and environmental protection is a top priority. Irrigation is the most widespread use of reclaimed water in developing countries as well as water-scarce regions of the developed countries.

When reclaimed water uses have a potential route for human exposition, the main concern is the health risk associated with exposure to biological contaminants including bacteria, helminths, protozoa, and enteric viruses (Mujeriego and Asano, 1999).

The quality requirements for reclaimed water depend on its final specific application and should be taken into account when the type of treatment is selected. Such requirements can be classified into three groups (De Koning et al., 2008):

• Related health issues, such as the spread of microorganisms in aerosols.

• Related operational aspects, such as plugging, fouling, corrosion, and pressure drops, leading to malfunction of process equipment.

• Related public acceptance or users, such as colored water, with scents and flavors.

In this work the water reclaimed uses for agricultural irrigation and industry process, and their associated sanitary risks have been considered in more detail because they are related with the developed research work.

2.3.2. Reclaimed water use for agricultural irrigation

Currently, regenerated wastewater is widely used for urban and agricultural irrigation. Approximately 70% of global water demand is associated with agricultural production (Lazarova and Asano, 2005), so the potential for this reclaimed water use, especially in places where water resources are scarce, is high. Moreover, available water for agriculture is critical for food security. Agricultural irrigation includes the irrigation of both horticultural crops (raw consumption) and those with further processing (cereals, citrus, and grapes). The used irrigation techniques can be spray, micro-sprinkler, drip, and flood (Mujeriego, 2006). In Mediterranean countries, such as Spain, the treated wastewater at different levels (primary, secondary or tertiary) is mainly used in a direct or indirect way for irrigation. Concerns about human health and the environment are the most important restrictions in regard to the reuse of reclaimed water for irrigation (Fatta and Anayiotou, 2007). Some effects that should be considered are possible groundwater pollution, soil contamination, and the adverse effect on farmers or/and consumers of reclaimed water irrigated products.

Two important studies conducted in California in 1970-1980, one about Pomona virus and the other on wastewater regeneration in Monterrey, showed that a pathogen-free effluent can be obtained from a municipal wastewater treatment plant using advanced or tertiary treatment. This proves scientifically that even eaten raw food crops could be irrigated using reclaimed water without any adverse effects for the public health (Asano, 1998).

The degree of required treatment for wastewater, and the type of needed monitoring depends on the specific application where reclaimed wastewater will be used. For example, a highly degree of treatment will be necessary if raw-eaten vegetables are irrigated, or if the irrigation system includes human contact, such as spray irrigation.

The feasibility of using reclaimed water for irrigation should be evaluated based on various water quality factors besides the microbiological quality and its associated health risk such as salinity, trace elements, rate of infiltration into the ground, and other water quality criteria. For example, the quality of irrigation water has particular importance when it is used in arid areas where the evapotranspiration rates are high. Due to this process, the salts contained in the irrigation water are deposited and accumulated on the soil; so soil and crop properties must be taken into account when the reclaimed water use is planned. The possible problems, however, do not differ from those caused by salinity or the presence of trace elements in any other water source, and it is a concern only if they restrict the use of water or require special handling to maintain an acceptable crop production (Metcalf & Eddy, 1991).

Reclaimed water can increase crop growth and yield because its nutrients supply a fertilizer value to the crop. Ham et al. (2007) found that reclaimed water irrigation did not adversely affect the yield of rice; on the contrary, it was increased a 50% compared to the controls. Moreover, the chemical characteristics of the soil did not change significantly during the experimental period of irrigation. Other study have showed that the tomato crops were successfully grown on treated municipal wastewater-supplied plots, with higher yields (approximately 20%) than on plots supplied with fresh water (Cirelli et al., 2012). Therefore, in some cases the use of reclaimed water could lead to the reduction in uses of chemical fertilizer (Lazarova and Asano, 2005). However, if nutrients are in excess, this can cause various problems related to vegetative growth. For example, extra sodium can affect soil structure and may also reduce aeration (Rengasamy, 2010). Also, it is important to note that the presence of high concentrations of residual chlorine (>5 mg/L) can damage plants if reclaimed water is sprayed directly onto the foliage (Metcalf & Eddy, 1991). The presence of heavy metals usually is a little concern for irrigation using reclaimed water since they are efficiently removed during wastewater treatment (Sheik et al., 1987). Gao et al. (2013) examined the accumulation of heavy metals by soil irrigated with reclaimed water and found that the contents of heavy metals have no obvious differences between soils irrigated with reclaimed water and well water at 0~140 cm.

The use of reclaimed water for irrigation should not cause a deterioration compared to irrigation with surface water in order to achieve wider acceptance.

Regarding health risk, biological and chemical contaminants can be found in treated wastewater. Biological pollutants are limited by regulations that allow the safe use of reclaimed water. Standard regulations usually are based on the human exposure degree to regenerated water, but the potential effects of using this type of water in crops and soils are generally not considered (Metcalf & Eddy, 1991).

2.3.3. Reclaimed water use for industrial process

About 20% of global water demand is related to industrial use (Metcalf & Eddy, 1991). This percentage varies significantly; in Africa, for example, the use of water for industry accounts for 5%, while in Europe, industrial water consumption accounts for 54% of total consumption[1].

In many cases, the water used in the industry is of unnecessarily high quality for the use to which it is intended. The analogy in domestic water use is direct; for example, the use of drinking water for the toilet or watering the garden. Similarly, in industrial processes there are many applications that could use water of lower quality. This provides opportunities for recycling. Often, 50% or more of industrial water consumption is used in cooling processes (Kohli and Franken, 2011), a need that can be satisfied with a lower water quality.

Reclaimed water can be used in industrial application as long as its quality suits the specified requirements. Other aspects to consider in water reuse are the distance from the WWTP that supplies water to the industry and the availability and quality variability of the water that such plant supplies. The most common uses of reclaimed water in industry are cooling and

[1] http://portalsostenibilidad.upc.edu/detall_01.php?numapartat=5&id=24. Last access 10/09/2013.

power generation, followed by its use in heaters and rapid cooling. In such systems, the use of reclaimed water by industry relieves pressure on scarce water resources.

Cooling Towers

Cooling towers are one of the most commonly used cooling systems in the industry. They are used to lower the temperature of recirculated water used by condensers and heat exchangers in chemical plants, power plants, and air conditioners. Its working principle is simple and consists of putting in contact a hot liquid with an unsaturated gas which is commonly air. Thus some portion of the liquid flow evaporates (partially saturating the gas phase) decreasing its temperature.

The cooling towers are large diameter columns with special types of packing designed to provide good air-water contact together with low pressure drop. Hot water is distributed over the packing by means of spray nozzles or by a grid of slots through the pipeline. Air circulates through the packing forced by fans or induced by natural convection (McCabe and Smith, 2002).

In a cooling tower water vapor is lost as moist air at the top of the tower. However, the latter is negligible, since it corresponds to 0.005% of recirculated water (Metcalf & Eddy, 1991). Due to this water loss by evaporation, the salt content in the cooling water system increases. To avoid precipitation, some water is drained and replaced with water with low salt concentration to maintain the system volume and salinity. This water is called makeup water and it could be supplied by reclaimed water.

The water quality in cooling systems is important because it can lead to various operational problems caused by:

- *scaling and fouling*: is widely understood as the deposition and adhesion of various kinds of substances in a system. This reduces the diameter of the pipe, causing flow problems (increasing pressure drop along pipes). Most importantly it decreases the efficiency of heat exchange decreasing the overall heat transfer coefficients. These deposits can also cause problems of localized corrosion (forming small local concentration cells), therefore increasing facilities maintenance costs. The most abundant solid deposits are those of calcium salts, such as carbonates, sulfates, and phosphates. It is controlled through the use of dispersants.
- *metal corrosion*: occurs when an electric potential between different metal surfaces is created, and one of the metals oxidizes and dissolves into solution. Contaminants such as dissolved solids increase electrical conductivity generally accelerating corrosion processes. Problems caused by corrosion are economically important because the corroded material has to be replaced, and also because of the process down time generated for the required repairs. One way to control this problem is the use of chemical corrosion inhibitors.
- *biological growth*: heat and moisture present in a cooling tower makes it a propitious (favorable) environment to promote biological growth. Nutrients, particularly nitrogen and phosphorus, encourage the growth of microorganisms that can adhere and deposit on heat exchange surfaces, inhibiting heat transfer and water flow. In addition, certain microorganisms produce corrosive by-products during growth. The way to control these problems is the use of biocides, antifouling agents, and sulfuric acid, among others.

These series of problems arise when any type of water is used; however these issues worsen when reclaimed water is used due to the fact that it generally contains pollutants in greater concentrations. These sources contain two to five times greater amount of dissolved solids and higher concentrations of organic matter (Williams, 1982). Thus, their treatment may require larger quantities of chemical treatment agents (biocides, antifouling or inhibitors) (EPRI, 2003).

Due to the reasons and problems mentioned above, when reclaimed water is used in different industrial applications it must comply with different treatment requirements as shown in Table 2.2. It shows that the requirements of wastewater treatment for reuse in

cooling towers are not high if compared to other industrial uses such as process water, or boilers water. However, in order to be used, it has to ensure that public health is not compromised. Thus, the safe and adequate disinfection is one of the most critical objectives in any water reuse program.

Table 2.2. Requirements of wastewater treatment for reuse in different industrial uses (Asano and Visvanathan, 2001).

Industrial use	Nitrogen and phosphorus removal	Chemical precipitation	Filtration
Makeup water in cooling towers	Usually	Yes	Yes
Condensing turbine exhaust	Sometimes	Rarely	Sometimes
Cooling by direct contact	Rarely	No	Sometimes
Bearings and equipment cooling	Yes	Yes	Yes
Process water	Yes	Yes	Yes
Boilers feed water	Reclaimed water use is not recommended		
Washing water	Sometimes	Rarely	Yes

2.3.4. Reclaimed water use in the world

North America: The US is the country which has the reclamation facilities and reuse systems implemented on a largest scale. First steps in planned reclamation and reuse of wastewater were taken in 1912, being California the pioneer state in this field. Water reuse is practiced in 17 of the 53 states that make up the country. In this sense, during the year 1995, 1264 million cubic meters per year (Mm^3/year) of reclaimed water were used. This volume corresponds to 0.23% of withdrawn water to meet water demand for different applications. This percentage increased to 1.5% in 5 years (Asano et al., 2000). Regarding Canada, there is a growing interest in the reuse of wastewater, and particularly in the use of treated gray water in commercial and residential buildings. Nowadays, water reuse is generally practiced in small-scale or experimental basis (Exall et al., 2006).

Central America and South America: According to the drinking water and sanitary service assessment from the Pan-American Health Organization (PAHO)[2], Latin America and the Caribbean have approximately 479 million inhabitants, of whom 130 million - about 27% - lack of potable water household connections, 255 million (53%) have no connections to the sanitary sewer, and only about 86 million (18%) are connected to sanitation systems in good conditions. It is estimated that in Latin America more than 100 Mm^3 of domestic sewage are annually dumped to watercourses. About 400 m^3/s of raw sewage is poured into rivers and lakes, to be later used to irrigate about 500,000 hectares (Peasey et al., 2000). These former data show that the reuse of wastewater is almost nil in these regions, and when exists it is informal rather planned.

Europe: In the last decades the EU and its member states have implemented wide and national measures to ensure a sustainable water resource management. Thus, wastewater reclamation and reuse was promoted in the Water Framework Directive (Bixio et al., 2006). The European Environment Agency has informed that over the past 17 years significant progress in water sanitation has been reached[3]. However, this progress has been uneven in the different areas that make up the EU. The increase of the treatment capacity was significant for all member states except Sweden, Finland, and the Netherlands where this

[2] www.paho.org. Last access 06/12/2013.
[3] http://www.eea.europa.eu/ Last access 07/15/2010.

capability was already high. The most significant increase has reached in the southern countries like Spain and Greece.

Two percent of the treated water effluent is reused in Europe. Additionally, the reuse is increasing at a rate of 25% per year. Nowadays, there are more than 200 reclaimed water use projects in operation and others at different planning stages (Jiménez and Asano, 2008b).

The Mediterranean region is the area that, due to its hydro-conditions, needs to incorporate reuse of water into the balance of their water resources more urgently. However, very few countries have exploited all the reclaimed water use potential to date (Bixio et al., 2006).

Africa. In some countries of this continent, including Morocco and Algeria, more than half of the wastewater treatment plants are not working properly due to lack of maintenance. The population growth and increasing urbanization leave behind the development of sanitary infrastructure, being little effective the management of wastewater and urban waste (Bahri et al., 2008).

Asia: The planned water reuse on this continent is basically carried out in the islands, and arid and semi-arid areas. Japan recycles 49% of total reclaimed water for public-urban use (plant watering, gardens, parks, golf courses irrigation, and in some areas for toilet flushing), 31% for environmental uses (increasing river flow), 10% for industrial use (process and cooling) and only 8% in agriculture, presenting a situation contrary to other countries where the major uses are agricultural and industrial applications (Ogoshi et al., 2001).

China has made efforts to regenerate and reuse wastewater since the 80's. In 1999 12,800 Mm^3/year of wastewater were generated by this country. From those, 4,081 Mm^3/year (31%) were treated in 398 wastewater treatment plants, of which 272 are biological systems. Currently, reclaimed water is used mainly in agriculture, industry, public-use, and environment. An overview of reclaimed water use in China was recently published by Yi et al. (2011).

Reclaimed water has been regarded as a new source of water and has been incorporated into the water balance in Israel. This country, currently, reuses over 65% of total municipal wastewater produced in the country, and is planning to reuse more than 90% by 2020 (Brenner, 2012; Juanico and Friedler, 1999; Juanico and Salgot, 2008).

Oceania: Different and important actions related to wastewater regeneration and reuse have been undertaken in this region due to weather conditions and water shortages. Among these actions, it is important to highlight the ambitious strategic plan for wastewater regeneration and reuse of Queensland (QWRS, Queensland Water Recycling Strategy). Reuse is increasing at a rate of 10-17% per year in Australia, and important water reuse programs have been implemented to promote reclaimed water use (Jiménez and Asano, 2008b).

For further information about water reuse around the world, an interesting overview can be found in the book edited by Jiménez and Asano (2008a).

2.3.5. Use of reclaimed water in Spain

In recent years there has been an important increasing of the use of reclaimed water in Spain, together with the increasing number of WWTP in operation to fulfill the treatment requirements set in the EU directive 91/271/EEC (Marecos do Monte, 2007). During the year 2001, treated wastewater covered a 346 Hm^3/year demand; in 2004 the demand was 377.5 Hm^3/year, while in 2006 a reclaimed water flow of 450 Hm^3/year was used. This flow corresponded to 13% of the total reclaimed water flow (3,370 Hm^3/year) that is produced by the 2,533 existing wastewater treatment plants (Ortega de Miguel and Iglesias Esteban, 2007). Currently, 4,845 Hm^3/year of treated wastewater are produced in Spain, with a reuse rate of 10.14% (491.17 Hm^3/year) (EPSAR, 2012).

Among the possible causes attributed to the increased reuse of reclaimed water can be included:

- Regeneration promotion (Directive 91/271/EEC), which has allowed the increasing in the construction of treatment plants to have available reclaimed water near to the demand points (Ortega de Miguel and Iglesias Esteban, 2008).
- Deficit of hydric resources.
- Water resources degradation.
- More reliable reclamation technologies.
- Improved wastewater management.

The volumes of reclaimed water use in Spanish different geographical regions are described in the Table 2.3. It is noted that the reuse of wastewater in Spain is mainly concentrated in the Mediterranean coast and islands. It is not surprising, because of knowing that in both the Valencia and Murcia coasts there are a large collection of golf courses and there are a lot of crop fields also.

Reclaimed water is used predominantly for agricultural irrigation in Spain, as in many of the southern Europe countries (Bixio et al., 2006; Melgarejo, 2009; Ortega de Miguel and Iglesias Esteban, 2008). Data related to the reclaimed water different uses are shown in Table 2.4. Valencia and Murcia are the autonomous regions that mainly use reclaimed water for agricultural irrigation (McCann, 2012; Melgarejo et al., 2009). This type of use, as it was before mentioned, allow for ecological recovery. The irrigation of golf courses is the most important activity included in the recreational use (Melgarejo et al., 2009). It is worth noting that the percentage corresponding to industrial use is very low. However, as was highlighted before, this kind of use has a high potential, especially in chemical industry and thermoelectric power plants.

In spite of water reuse is already an essential and reliable water supply option for many regions in Spain, a model developed to evaluate the water reuse potential in Europe has shown that Spain is by far the country with the highest reuse potential (Hochstrat et al., 2005).

Table 2.3. Reclaimed water use in Spain distributed among regions. Adapted from EPSAR (2012).

Areas	**Volume (Hm^3/year)**
Valencia	156.1
Andalusia	123.5
Murcia	86.0
Balears Islands	34.8
Catalonian	33.2
Canarias Islands	31.7
Madrid	6.8
Basque Country	6.1
Castilla-La Mancha	4.9
Castilla and Leon	3.6
Asturias	1.9
Aragon	1.9
Galicia	0.6

Table 2.4. Type of reclaimed water uses in Spain (CEDEX, 2008).

USES	Percentage (%)
Agricultural irrigation	70.9
Ecological	17.7
Recreational	7.1
Urban irrigation	4.0
Industrial	0.3
TOTAL	**100**

2.3.6. Reclaimed water use in Catalonia

In Catalonia, 692 Hm^3/year of wastewater are reclaimed. An important part of this volume is poured into the sea, while 4.8% is used indirectly (EPSAR, 2012). The main applications are environmental or ecological use (76%), recreational use (14.5%), and agricultural irrigation (7.8%)[4]. With the ongoing and planned activities for the coming years, the Agència Catalana de l'Aigua (Catalonian Water Agency) wants to increment the water reuse rate from 5% to 31% by the year 2015. Moreover, WWTP are planned to be built to treat wastewater for all villages with less than 2,000 inhabitants.

In terms of reuse, it is important to highlight the great work that has been done by the Consortium of the Costa Brava (CCB) over the past 20 years. Studies conducted since 1985 by the Polytechnic University of Catalonia in collaboration with the CCB and other public and private entities have helped to establish a reclaimed water management system in the CCB (Mujeriego, 1998).

The CCB brings together over twenty municipalities and dedicates its activity to both water supply and reclamation and reuse of wastewater. The creation of the CCB had the objective to alleviate an economic necessity linked to tourism development in the area.

2.4. Reclaimed water regulations and guidelines

The implementation of a water reclamation project has two essential and complementary requirements:

1) set appropriate quality levels for each of the possible uses of reclaimed water;

2) define the extent of needed wastewater treatment, and establish the numerical limits for effluent quality.

The development and approval of these technical aspects of water reclamation are generally the most controversial aspect of any water reuse program. This is mainly due to the difficulty of establishing a causal relationship between water quality and potential health and environmental effects. Proof of the former is the great diversity and heterogeneity of water quality criteria that can be found in the regulations and guidelines established by various countries and international organizations (USEPA, 2004; World Health Organization, 1989).

To protect public health, considerable efforts have been made in order to establish guidelines and regulations for the safe use of reclaimed water. These standards focus mainly in sanitary criteria and do not take into account the treatment technology, water application mode, or the potential effect of reclaimed water in the environment. However, it is important to note

[4]http://aca-web.gencat.cat/ Last access 08/12/2013.

that FAO has published a guideline considering the reclaimed water effects on soils (Westcot and Ayers, 1985), as well as many studies have been performed taking into account the effect of treated wastewater irrigation on soils and crops (Farah and Batarseh, 2008; Pedrero et al., 2012). Also, the presence of microcontaminants- such as pesticides, surfactants, disinfection by products, and human and veterinary pharmaceuticals among others- should be taken into consideration (Calderón Preciado et al., 2013; Daughton, 2004; Fatta Kassinos et al., 2011; Shenker et al., 2011).

The first regulations for wastewater reuse were enacted in 1918 by the State of California in the US (Asano and Levine, 1996). This legislation evolved into the Title 22 standards (Jiménez and Asano, 2008b). Towards the end of the twentieth century, the benefits of promoting the reuse of reclaimed water to supplement water resources have been recognized by many state legislatures in the US, as well as by the EU. For example, in 1970 the California Water Code established that *"is the intention of the Legislature that the State assumes all possible steps to promote the development of water reclamation facilities so that reclaimed water is available to cover growing water requirements"* (Asano, 1998). In the same context, the Commission of the European Communities (Directive 91/271/EEC) stated that "*treated wastewater should be used when appropriate. The disposal routes should minimize adverse effects on the environment"* (Asano, 1998).

Alternative regulations related with water reuse, mainly for water irrigation, have been established by the World Health Organization initially in 1989 and revised in 2006, such as the "Guidelines for the safe use of wastewater, excreta and graywater. Volume 2: Wastewater use in agriculture"[5].

In 1992 the US Environmental Protection Agency (USEPA) published a document named "Guidelines for the reuse of reclaimed wastewater". But in spite of this document, in US each state defines its specific criteria for the use of reclaimed water. The main differences between them rely on the monitoring requirements (total or faecal coliforms, enteric viruses) for each water use (Asano, 1998). Recently, the USEPA has published the 2012 EPA Guidelines[6].

In developing countries, the water quality criteria, as far as wastewater reuse is concerned, reflect a complex balance between public health protection and limited financial resources available for public works and health systems.

The use of reclaimed water requires the adoption of measures to protect public health as well as environment. Although some countries have already established wastewater reuse norms, more work still needs to be done to define international and standards regulations in order to solve the lack of clear criteria on when to reuse and on quality standards, and to increase the public acceptance of reclaimed water as a reliable water resource.

2.4.1. Reclaimed water regulations in Spain

The regulations related with the wastewater management in Spain include:

- Royal Decree-Law 11/95 that establishes the regulations applicable to the treatment of urban wastewater. The main objective of these regulations was to protect the surface water from untreated wastewater discharge. To accomplish the regulations, different infrastructures for wastewater treatment were developed.
- Water reuse regulations in Basin Hydrological Plans: Tajo (1999) and Guadalquivir (1999) (Iglesias Esteban and Ortega de Miguel, 2008).
- Royal Decree-Law 1/2001 that approves the revised text of the Water Act. It refers to the fact that government should set the basic conditions for the wastewater reuse as well as establish the required quality of treated wastewater (Iglesias Esteban and Ortega de Miguel, 2008).

[5] http://www.who.int/water_sanitation_health/wastewater/gsuww/en/. Last access 08/10/2013.
[6] http://www.watereuse.org/government-affairs/usepa-guidelines. Last access 08/10/2013.

- Standards and recommendations established in some regions: Catalonia (1994-2005), Baleares (1995-2001) and Valencia (2004) (Iglesias Esteban and Ortega de Miguel, 2008).
- Royal Decree-Law 1620/2007 (RD1620/2007) which sets the legal framework for the reuse of treated water in Spain.

The RD 1620/2007 is the most relevant reclaimed water regulation in Spain. It refers to the permitted uses for reclaimed water in the Chapter II, Article 4 and Annexes. They are grouped into five major sections: urban, agricultural, industrial, recreational, and environmental. The criteria of quality and numerical acceptable levels established for each specific reuse application are defined in Article 5. Biological and physicochemical variables were considered. The other chapters refer to the procedures required to obtain licenses to use reclaimed water. Table 2.5 summarizes the main water quality criteria required for reuse in agriculture or industrial cooling.

It is important to note that the absence of *Legionella* spp. for industrial cooling use is required. For other industrial uses, a *Legionella* spp. concentration of 100 colony-forming units (CFU)/L is admissible. In addition, an approval emitted by the health authority of the specific facilities control program (according to the Royal Decree 865/2003, which sets the hygienic-sanitary criteria for the prevention and control of legionellosis) will be required. Moreover, those industries that use reclaimed water in their cooling towers should not be located in urban areas or near places where public or commercial activities are developed.

The RD 1620/2007 also establishes water sampling frequencies. The analytical control of *Legionella* should be done three times per week for reuse in cooling towers, as it should be fortnightly for agricultural irrigation use.

Table 2.5. Quality requirements for industrial and agricultural uses of reclaimed water according to the RD 1620/2007.

Water uses	Maximun Admissible Value (MAV)				
	Nematode intestinal eggs (eggs/10 L)	***Escherichia coli*** **(CFU/100 mL)**	**Suspended solids (mg/L)**	**Turbidity (NTU)**	***Legionella*** **spp. (CFU/L)**
Agricultural					
Quality 2.1 Eaten raw crop irrigation	1	100	20	10	1,000, when aerosolization can happen
Quality 2.2 Crop irrigation	1	1,000	35	Not fixed	
Industrial					
Quality 3.2 Cooling tower and evaporative condensers	1	Absence	5	1	Absence

NTU: Nephelometric Turbidity Units. CFU: Colony Forming Units.

2.5. Water quality monitoring

The safe use of reclaimed water implies reducing the public health concerns to acceptable levels. This means that the risks are low enough that the water reuse has no practical consequences for a specific population. Thus, proper surveillance and monitoring of microbiological water quality are needed. This is in agreement with the idea expressed by Stratton and Matthews (2009) who clearly said that "the ability to directly detect pathogens using the most accurate techniques will be critical for water managers and providers to confidently assess and manage risks of existing and new water sources".

Since monitoring for all possible microbial constituents is not realistic and uneconomical, the microbiological water quality is commonly estimated or monitored using specific target organisms, called microbial indicators, which correlate with the presence of a pathogen or class of pathogens. These indicators suggest the presence of pathogens (e.g. *Escherichia coli* indicate the presence of faecal origin pathogens) or/and are representative of the water treatment efficacy (e.g. total heterotrophic bacteria or total coliforms).

An ideal indicator organism should mimic the behavior and characteristics of the target pathogenic organism and, at the same time must be easier and faster to isolate and detect. Furthermore, its identification should not entail a serious health threat to laboratory workers (Keegan et al., 2009; Metcalf & Eddy, 2007). Since human and animal feces are the greatest source of human waterborne pathogens, faecal indicators, such as faecal coliforms and more specifically *Escherichia coli*, have been used as the most acceptable indicators of water and wastewater quality. Although the microbial indicator use has been effective, simple, and allows control under the current regulations (RD 1620/2007), there exist some limitations because it is unlikely that all pathogenic organisms (bacteria, protozoans, viruses, and helminths) behave in the same way and can be represented by the current indicators (Codony et al., 2009). It is important to bear in mind that wastewater is a complex matrix which is faecally contaminated by default (Keegan et al., 2009), so the presence of faecal indicators is not a confirmation of pathogens presence. Moreover, the current indicators are in general bacterial and they are usually more sensitive to disinfection than viruses and protozoan cysts, so the absence of an indicator organism is not always a guarantee of the pathogen absence (Lemarchand et al., 2004). Many studies have demonstrated poor correlation with indicator organisms and pathogenic organisms in treated wastewater (Agulló-Barceló et al., 2012; Baggi et al., 2001; Costán-Longares et al., 2008; Harwood et al., 2005). These studies suggest that the use of a single indicator or/and the currently used indicators is not enough to protect adequately the public health, so research work to improve pathogen detection or improved indicators is needed. Monitoring a combination of traditional and new indicators, as well as specific and direct pathogen monitoring in same cases seem to be a good choice for ensuring the microbial quality of reclaimed water.

Generally the analysis of microorganisms in water involved some common steps like sampling, concentration, detection, and quantification. Samples must be representative, and appropriate conservation conditions should be ensured right from the sample collection until the end of the analysis procedure. An important limiting factor in the assessment of water quality often is the low number of each microorganism present; therefore a concentration (e.g. filtration, centrifugation, adsorption-elution, etc.) of the sample is usually performed before the detection and quantification steps. Conventional pathogen detection includes culture methods for bacteria, cell culture or plaque count technique for viruses, and microscopic methods for protozoa (Lemarchand et al., 2004). However, the determination and quantification of microorganisms can be performed by using different technics like microscopic and other optical or imaging methods, culture on media or in living hosts, viability or activity measurement, immunoassays, and nucleic acid assays. Often, several of these assays are combined or used concurrently in order to provide more information on the quantity, identity, characteristics and state of the target organisms (National Research Council, 1999). Fluorescent detection methods, including flow cytometry and microscopy observation, are able to assess microorganism viability by measuring intracellular pH, respiration, enzyme activity, and membrane integrity among others (Cenciarini et al., 2009).

However, in order to achieve species-specific determination, these techniques frequently need to be used in conjunction with other techniques (Cenciarini-Borde et al., 2009). Moreover, background and self-fluorescence problems can be found when they are applied (Cao-Hoang et al., 2008).

Although all the above mentioned techniques deserve special mention, culture techniques and nucleic acid-based methods (particularly qPCR) will be described below because the present research work is mainly focus on the bacterial detection in water using culture and qPCR, and on the improvement of real-time PCR application especially in the analysis of environmental samples.

2.5.1. Culture techniques

The established or standard method for the detection of microorganisms in water and wastewater is based on culture. This method relies on the growth of microbial organisms in a nutritionally rich culture media and under controlled laboratory conditions that can satisfy their physiological requirements. It is important to note that the nutritional requirements as well as the optimal physical and chemical conditions differ for each microorganisms or group of microorganisms, so selective nutritious broth or agar media and environmental conditions are currently used to grow, isolate, or/and enumerate the target organism while simultaneously suppressing the indigenous background flora. Microorganism culture can be performed in liquid (broth) or solid (agar) media.

Commonly used techniques based on culture, especially bacteria cultivation, are the Most Probable Number (MPN) and plate count. The MPN method is a semi-quantitative assay that consists of inoculating a series of tubes with appropriate decimal dilutions of the water sample. Production of gas, acid formation or abundant growth in the test tubes after an incubation step at a determined temperature constitutes a positive presumptive reaction. This method has important limitations especially the lack of precision, so it has been replaced in many instances for plate count (Rompré et al., 2002).

Direct plate counting can be performed via three different procedures: spread plate method, pour plate method, and membrane filtration plate count method.

The spread plate method consists of evenly spreading the diluted sample over an agar plate. A volume no higher than 0.1 mL of the diluted sample should be used, otherwise the agar could not be able to absorb the sample excess. Using this method, colonies that form on the agar surface can be counted (Madigan et al., 1997).

When pour plate method is performed, a diluted sample is pipetted into a sterile Petri dish, then melted agar is poured in and mixed with the sample. This method allows for counting bacteria present in a larger volume of the diluted sample (0.1 – 1 mL). Colonies formed throughout the agar, and not only on the surface are yielded. Caution must be taken with this method to ensure that the organism to be counted can withstand the temperatures associated with the melted agar (Madigan et al., 1997).

Membrane filtration plate count method allows for analyzing large sample volumes (100 mL), and it is one of the worldwide standard methods used to determine indicator organisms in water. This method is similar to the spread plate method since consists in filtering a known amount of water sample on a sterile filter with a specific pore size to retain the target organisms, and then the filter is incubated on a medium. The colonies that growth on the filter are then enumerated (Rompré et al., 2002).

Liquid enrichment and posterior confirmation and/or isolation on solid media is needed for some microorganisms when their concentration is too low compared to the amount of non-pathogenic microorganisms or background micro-flora. Specific methods are required for virus and protozoa detection because these organisms grow differently. Besides several enteric viruses and protozoa cannot be cultured in the laboratory (Lemarchand et al., 2004).

In some cases the identity of the cultured bacteria is confirmed by one or more of several methods, like subculturing on other differential and selective media; biochemical, metabolic

and/or other phenotypic analyses (for substrate utilization or conversion, enzyme activity, oxidation and reduction reactions, antibiotic resistance, motility, etc.); immunological analyses (e.g., serological, immunofluorescent, enzyme-immune, or radio-immune assays); or nucleic acid or genetic analysis (National Research Council, 1999).

From a public health and risk assessment standpoint the most relevant microbial detection methods are those can assess the infectivity of microorganisms. It is for that reason that culture-based techniques, which demonstrate that the target microbe is alive and capable of replication, are preferred and have been successfully used for an extended period of time. However they have some limitations that, in the context of a highly technical world, allow for questioning if a culture based method is still the most adequate microbial monitoring tool.

Only a very small fraction of waterborne organisms can be selectively cultured with currently employed growth media since artificial homogenous media typically fail to reproduce the ecological niches and the symbiotic relationships encountered in complex natural environments (Nocker et al., 2009; Sanz and Köchling, 2007). Moreover, injured or stressed cells or organisms which are viable but have lost culturability (known as viable but not culturable cells (VBNC)) cannot be detected by culture methods (Nocker et al., 2009). Oliver (2005) has pointed out that, although further studies are necessary to understand the importance of VBNC cells in the initiation of human infection, it appears that cells in this state retain virulence, and should be considered by researchers and government regulators involved in public health and risk assessment decisions.

Other important limitation of cultivation techniques is the time required for analysis. Depending on the growth rate of the organisms, the determination of viable culturable microorganisms normally requires days to weeks (Nocker et al., 2009). For instance *Legionella* spp. has a slow growth rate and it usually requires 10 to 14 days of incubation, although colonies can be observed after 72 to 96 hours. If the detection method is time-consuming, the opportunity to deal with water quality problems, and take decisions in a timely manner will be limited.

Legionella is also a good example for other drawback of culture-based technique that is the poor sensitivity when microorganism detection is hidden by the overgrown of faster growing bacteria. This could also occur in a sample contains microorganisms that may inhibit the target organism growth (Kao et al., 2013).

Above mentioned limitations of the culture-based methods led in the last two decades to the development and improvement of nucleic acid-based methods, whose implementation is growing rapidly in the different fields of microbiology, in particular in environmental microbiology (Lemarchand et al., 2004).

2.5.2. Molecular methods

Molecular methods targeting nucleic acids, in general do not require that microorganisms are cultured in order to detect them. These methods present an alternative or complement the conventional culture methods allowing for increasingly rapid detection. These methods include: hybridization (gene probe), nucleic acid amplification by PCR and other methods, restriction enzyme fragment length analyses (Restriction Fragment Length Polymorphism, RFLP), cloning, and nucleotide sequencing. Among these different nucleic acid-based methods, those based on nucleic acid recognition and amplification, such as PCR, have proved to be valuable tools for microbiology monitoring. They allow for detecting and characterizing microorganisms in different environments, and thus provide with better understanding of them.

Quantitative PCR is based on the revolutionary PCR method developed by Kary Mullis in 1985 (Mullis, 1990) and it is being used in a rapidly number of applications.

PCR involves the enzymatic amplification of a specific DNA region. Genomic DNA, usually obtained after an extraction and purification step, is exponentially amplified by a DNA polymerase using specific primer molecules (Schrader et al., 2012). There are several sources describing the basic of this technique. Mackay et al. (2007) explained that PCR

methods utilize a pair of synthetic oligonucleotides (short, single-stranded pieces of DNA) called primers, that hybridize in a 5' to 3' orientation to one particular strand of a double-stranded DNA target. Each hybridized primer forms a starting point for the production of a complementary DNA strand via the sequential addition of deoxynucleotides using recombinant DNA polymerases derived from thermophilic bacteria (*Thermus aquaticus*). The primer pair spans a target region that is exponentially and precisely replicated during the subsequent reaction cycles, that is the reaction product and it is usually called "amplicon".

Summarizing, a PCR cycle includes three steps:

- *denaturation*: single stranded DNA is obtained by heating (above 90 ºC) the targeted double stranded DNA, thus each single stranded DNA becomes a template.
- *annealing*: using a specific temperature (which is commonly tuned for each gene target, generally between 55 to 65 ºC) the primers hybridize or anneal to their complementary sequence on each template.
- *elongation*: at an optimal temperature, the DNA polymerase extends the primers using the provided nucleotides. It is important to note that DNA polymerase from *Thermus aquaticus* (Taq) is active over a broad range of temperatures (Innis et al., 1988). For that reason a PCR format requiring only two steps (denaturation, and a combined annealing extension step) is also popular. After each cycle, the newly synthesized DNA strands serve as templates for the next cycle.

Conventional PCR or end-point PCR uses agarose gel electrophoresis to separate and identify the PCR reaction products. The technique is mainly qualitative, since the presence of bands of the correct size on agarose gel allows for presence-absence conclusions, but the starting number of DNA targets can only be poorly estimated using the band intensity.

In contrast to conventional PCR, qPCR permits to detect and quantify the initial DNA concentration of the microorganism target in "real" time. The first study of qPCR (originally called "kinetic PCR") was published in 1993 by Higuchi et al. (1993). They demonstrated that a continuous monitoring of the level of amplified DNA over the course of the amplification, rather than in the end, could provide quantitative information as well as information about the amplification process itself which is useful for the optimization of PCR conditions.

Quantitative PCR uses the increase in the fluorescence of fluorogenic chemistries that results from its direct or indirect interaction with the amplicon. The initial DNA template concentration is directly related to the kinetic of fluorescence accumulation during the reaction. The fewer cycles necessary to produce a detectable fluorescence, the greater the number of target sequences (Higuchi et al., 1993; Nocker et al., 2009).

Nowadays there exist a great number of qPCR equipments (thermocycling instruments) paired with appropriate software to analyze rapidly and conveniently the PCR results. The data analysis methods, generally, are based on determining the threshold cycle (Ct) value, which is defined as the cycle number where the fluorescent signal rises above the threshold fluorescence (Nocker et al., 2009; Ramakers et al., 2003). The Ct is inversely proportional to the amount of target nucleic acid in the sample (i.e., the lower the Ct value the greater the amount of target nucleic acid in the sample). This approach ensures that interfering factors associated with later stages of the amplification are minimized and considerably improves the determination precision (Rasmussen, 2001). Thus, if the Ct values of standards of known DNA concentration are used to create a regression line or a calibration curve (usually called standard curve), the starting DNA concentration of a target organism can be estimated in unknown samples. This method theoretically assumes that all samples and standards have approximately equal amplification efficiencies (Souaze et al., 1996). However, it is important to note that qPCR efficiency is driven by the amplification process; the theory indicates that DNA duplicates in each cycle so a constant efficiency equal to 2 is assumed. Nonetheless, different factors which are related with reagents and primers performance, methodological procedure, and sample DNA quality, frequently, have a considerable impact on DNA amplification. Consequently, the efficiency value is found to be lower than the theoretical one of 2. More importantly the former factors are also prone to improvement and, as Rodriguez

Lázaro and Hernández (2013) mentioned, the efficiency of a qPCR will vary according to the degree of method optimization that has been carried out.

Regarding the fluorogenic chemistries, different fluorescence techniques can be used in qPCR. They can be classified in two groups, non-specific DNA binding dyes or sequence specific probes. Detailed information regarding them can be found in the literature (Mackay, 2007; Rodriguez Lázaro and Hernández, 2013; Valasek and Repa, 2005).

SYBR Green I is a non-specific dye that binds double-stranded DNA, and upon excitation emits light. Its fluorescence when it is bound is above 1000-fold higher than when is free (Morrison et al., 1998), so fluorescence measurements should be performed at the end of the elongation step of every qPCR cycle. It is excited at 480 nm and its emission spectrum has a maximum at 520 nm (Jin et al., 1994). Non-specific intercalating dyes are a low cost and easy to use option, because they can normally be used with any target and any pair of primers. However, this advantage is also a disadvantage, since the specificity only depends on the primers when SYBR Green is used. Thus, multiple double-stranded species that may be present, such as primer-dimer and non-specific amplification products, cannot be discriminated, leading to confusion in the interpretation of results, as well as producing overestimation in the microorganism quantification. This confusion and overestimation is especially important when the microorganism target is present in samples at low concentration. Nevertheless, desired products can be distinguished from undesired products by using a melting curve analysis, where fluorescence is plotted as a function of temperature. This analysis can be used to differentiate amplification products separated by less than 2 °C in melting temperature. The shape and position of the melting peaks (melting temperature) are functions of the GC/AT ratio, length, and amplicon sequence (Ririe et al., 1997). Melting temperature is defined as the temperature at which 50% of the oligonucleotide-target duplexes remain hybridized (Mackay et al., 2007). SYBR Green has been successfully used in real time PCR to detect microorganisms in wastewater and reclaimed water (Chetta et al., 2012; Dong et al., 2010; Ferrer et al., 2012; González et al., 2010; Nayak and Rose, 2007).

Sequence-specific probe based methods rely in the use of one or more fluorescently labeled oligonucleotides that are positioned between the two primers, thus these methods can only detect the presence of a specific amplicon within the reaction. These probes can be classified in two groups: hydrolysis probes, and hybridization probes (Rodriguez Lázaro and Hernández, 2013). Hydrolysis probes, commonly named Taqman probes, are dual labeled with a fluorescent reporter dye at the 5′ end and a quenching dye at the 3′ terminus. Additionally, the probes must be blocked at their 3′ end to prevent the extension during the annealing step. Whilst the probe is intact, the quencher molecule absorbs the natural fluorescence emission of the reporter dye by Forster-type energy transfer (Fluorescence Resonance Energy Transfer, FRET). During the extension step, Taq polymerase enzyme with 5′ exonuclease activity digest the probe freeing the reporter from the quencher, and thus, the reporter fluorescence can be detected (Abd-Elsalam, 2003; Arya et al., 2005; Rodriguez Lázaro and Hernández, 2013). In contrast to hydrolysis probes, hybridization oligoprobes are not destroyed to produce a fluorescent signal. In this case, fluorescence is generated by a change in the secondary structure of the probe during the hybridization phase, which results in an increase of the distance separating the reporter and the quencher dyes. The most important probes in this group are those containing hairpins (Molecular Beacons, Scorpion primers, etc.), and the FRET probes (Mackay et al., 2007; Rodriguez Lázaro and Hernández, 2013). It is important to note that TaqMan is the most widely used qPCR assay, and it has been used for multiple purposes including microbial quality monitoring in water samples (Chatzisymean et al., 2011; Shannon et al., 2007; Spano et al., 2005).

Multiple detection probes can be used to detect multiple targets, and thus the set-up time, the amount of reagent used, and the thermal cycler demand are reduced. However, a multiplex assay presents technical challenges due to possibility of competition for the reagents and the increased complexity of the primers mixes, since primer dimer formations and other primer interactions can also occur. According to Nocker et al. (2009) the impact of this technology on waterborne pathogen detection remains to be seen.

Compared to conventional PCR, major advantages of qPCR are the wide dynamic range of quantification (Morrison et al., 1998), the close-tube format that avoids the risk of PCR products carry-over contamination into subsequent reactions, and the speed (Boyer and Combrisson, 2013; Higuchi et al.; 1993).

Nowadays the need for more rapid, sensitive and specific tests is essential in the water management. Rapidity, versatility, specificity, and sensitivity are considered as important attributes of qPCR (Nocker et al., 2009). However, at the moment, there are still some limitations to overcome before the introduction of such methods in routine analysis as standard methods.

Although sensitivity is commonly perceived as an advantage of qPCR methods, PCR sensitivity can hardly compete with cultivation methods if the work-flow is considered. It is important to take into account when a qPCR protocol is designed that sensitivity is further reduced by the template preparation (sample concentration and nucleic acid extraction). Moreover, a small aliquot of the extracted DNA is used in the reaction tube resulting in further loss of assay sensitivity. Sensitivity can also be reduced by amplification inhibition (Codony et al., 2009; Nocker et al., 2009).

The PCR is an enzymatic reaction and therefore is prone to inhibiting substances which may be present in the analyzed sample and which may affect the efficiency of the assay or even lead to false-negative results. In water samples the most common inhibitors are humic acids and other phenolic compounds, heavy metals, polysaccharides and urea (Nocker et al., 2009). A variety of inhibition mechanisms can occur depending of the inhibitory substance (Opel et al., 2010). These mechanisms can be linked to precipitation of DNA, denaturation of DNA, Taq Polymerase inhibition, binding to target DNA or the DNA polymerase, and cell lysis problems among others (Nocker et al., 2009; Opel et al., 2010; Rossen et al., 1992). In many cases inhibition problems may be avoided by using improved extraction-purification methodologies (McCartney et al. 2003), for instance through the use of commercially available extraction kits (Lievens et al., 2005). However, some of the reagents used in DNA extraction can be a source of inhibitors (Besetti, 2007; Peist et al., 2001). The DNA dilution approach can be other easy to apply method to handle PCR inhibition (Rajal et al., 2007), as well as the addition of amplification facilitators in the reaction, such as bovine serum albumin, T4 gene 32 protein, and betaine (Al-Soud and Radström, 2000). The use of internal amplification controls is highly recommended to assess for inhibition and avoid false negative results (Hoorfar et al., 2004; Wilson, 1997).

The need for expensive reagents and instrumentation has been pointed out as a disadvantage of qPCR technique (Mekata et al., 2012; Wong and Medrano, 2005). But, as with all new technologies, continuous advances in instrumentation, chemistry, enzymology, as well as the development of commercially available purification and detection kits have helped to reduce the costs and will help performing experiments with high-throughput/automation, thus increasing reliability of results and meeting industrial demands (Boyer and Combrisson, 2013; Girones et al., 2010). On other hand, the introduction of new reagents, chemistries, protocols, instruments, etc., makes difficult to compare results obtained in different laboratories, and setup standard protocols for specific microorganisms and determined matrices. Standardization is a key step in order to use qPCR technique in routine water quality monitoring analysis. Considering that, Bustin et al. (2009) have recently proposed some recommendations in a set of guidelines describing the minimum information necessary to evaluate qPCR experiments (MIQE). Their implementation can be important for the maturing of qPCR into a robust, accurate and reliable nucleic acid quantification technology (Bustin, 2010). It is important to note that some steps toward qPCR technique standardization are being given, for instance in 1999, the European Commission approved a research project (FOOD-PCR (http://www.PCR.dk)) which aims to validate and standardize the use of diagnostic PCR for the detection of pathogenic bacteria in foods (Malorny et al., 2003) , and in 2004 the U.S. EPA has developed a guidance manual for laboratories performing PCR analyses on environmental samples (Quality Assurance/Quality Control Guidance for laboratories performing PCR analyses) (Nocker et al., 2009).

Regarding qPCR validation and standardization, it is important to note that in spite of qPCR results being validated using culture results, both methods use different reference units to quantify microorganisms. Culture techniques have as reference the colony forming units (CFU) while the reference in qPCR is the gene copy number. Although the CFU and the gene copy number are related, there not exists a direct correlation between them. It is important to have this in mind when result analysis is performed (Codony et al., 2009).

Inability to discriminate dead/viable cells is, without doubt, one of the major drawbacks of PCR-based methods, such as qPCR. This leads to an overestimation of viable cells or even to false-positive results. For water quality monitoring and for the evaluation of disinfection treatments efficiency, accurate and powerful tools with the ability to discriminate viable from dead bacteria are needed and essential (Cenciriani-Borde et al., 2009; Nocker and Camper, 2006).

The intrinsic instability of RNA makes it a suitable candidate to be used as a viability indicator (Bentsink et al., 2002; Novak and Juneja, 2001). However, reproducibility in determining viable and dead cell counts accurately is difficult using mRNA as target (Sheridan et al., 1998). Working with RNA is technically more challenging than with DNA due to the risk of RNA degradation by ubiquitous RNases, and the storage instability of appropriate standards. Practical problems of extracting detectable levels of intact RNA from small numbers of bacteria and the risk of DNA contamination add complexity of RNA-based approaches and are impediments to its utilization in routine diagnostics (Nocker and Camper, 2006). According to Cenciriani-Borde et al. (2009), DNA is a good molecule for specific bacterial detection and quantification because it is present in only one or two copies per cell, depending on the replication activity, which allows for more reliable quantification, and its robustness after extraction and purification avoids false negative results.

A promising strategy to detect and quantify only viable cells by using qPCR, named viability PCR (v-PCR), has been developed by Nogva et al. (2003) and Nocker et al. (2006). The technique is based on sample treatment with photoactivatable, and cell membrane impermeant, nucleic acid intercalating dyes such as ethidium monoazide (EMA) or propidium monoazide (PMA) followed by light exposure prior extraction of DNA and amplification. Light activation of DNA-bound dye molecules results in irreversible DNA modification and subsequent inhibition of its amplification. Proof of concept of this new method and its reliability was attested by applying it on the detection of different microorganisms (Agusti et al., 2010; Brescia et al., 2009; Cawthorn and Witthuhn, 2008; Delgado Viscogliosi et al., 2009; Fittipaldi et al., 2010; Fittipaldi et al., 2011; Rawsthorne et al., 2009; Sanchez et al., 2012, Shi et al., 2012; Soejima et al., 2007), and on different matrices, including samples from complex environments like sludge from an anaerobic digestion plant, and wastewater (Bae and Wuertz, 2009; Varma et al, 2009). Despite the method success, further optimization is necessary in order to reach a complete suppression of dead cell signals (Nkuipou-Kenfack et al., 2013); since the resulting false-positive results leads to an overestimation of live cell population, which is especially problematic for the use of qPCR in water quality monitoring. Throughout the dissertation more detail information about this technique is included.

Taking into account the above mentioned advantages and drawbacks of the qPCR technique, further studies, optimization, and development of qPCR methods are necessary to fully support the use of these techniques in routine water monitoring analysis. Accuracy in determining the actual presence of infectious microorganisms will allow correct validation of disinfection treatment, enabling right decisions related with health and water management, and also will avoid false alerts, and the subsequent loss of public acceptance.

2.6. Reference

Abd-Elsalam, K.A. 2003. Non-gel based techniques for plant pathogen genotyping. Acta Microbiol. Pol., 52, pp. 329-341.

Agulló-Barceló, M., Oliva, F., Lucena, F. 2012. Alternative indicators for monitoring *Cryptosporidium oocysts* in reclaimed water. Environ. Sci. Pollut. Res. Int., 20, pp. 4448-4454.

Agusti, G., Codony, F., Fittipaldi, M., Adrados, B., Morató, J. 2010. Viability determination of *Helicobacter pylori* using propidium monoazide quantitative PCR. Helicobacter, 15, pp. 473-476.

Akpor, O.B., Muchie, M. 2011. Environmental and public health implications of wastewater quality. Afr. J. Biotechnol., 10, pp. 2379-2387.

Al-Soud, W.A., Radström, P. 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces and meat. J. Clin. Microbiol., 38, pp. 4463-4470.

Angelakis, A.N., Spyridakis, S.V. 1996. The status of water resources in Minoan times. A preliminary study. In: Angelakis, A., Issar, A. (Eds.). 1996. Diachronic climatic impacts on water resources with emphasis on Mediterranean region. Springer. Berlin, Germany, pp. 161-191.

Anonymous. 1994. Legionnaires' disease associated with cooling towers-Massachusetts, Michigan and Rhode Island. M.M.W.R., 43, pp. 491-499.

Armon, S., Starosvetzky, J., Arbel, T., Green, M. 1997. Survival of *Legionella pneumophila* and *Salmonella typhimurium* in biofilm systems. Water Sci. Technol., 35, pp. 293-300.

Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N., Patel, H.R.H. 2005. Basic principles of real-time quantitative PCR. Expert Rev. Mol. Diagn., 5, pp. 209-219.

Asano, T., Levine, A. D. 1996. Wastewater reclamation, recycling and reuse: past, present, and future. Water Sci. Technol., 33, 10–11, pp. 1–14.

Asano, T (Ed.). 1998. Wastewater reclamation and reuse. CRC Press. www.crcpress.com. US.

Asano, T., Ogoshi, M., Suzuki, Y. 2000. Lessons learned from the Japanese water reuse experiences, Water Reuse 2000 Conferences, San Antonio, Texas, January 30 – February 2. In: Kamizoulis, G., Bahri, A., Brissaud, F., Angelakis, A.N. 2003. Wastewater recycling and reuse in practices Mediterranean region: recommended guidelines. MED-REUNET Mediterranean Network on Wastewater Reclamation. www.med-reunet.com/docsupload/medrecomguidelines.pdf.

Asano, T., Visvanathan, C. 2001. Industries and water recycling and reuse. Business and industry-A driving or braking force on the road towards water security. Founders Seminar, Stockholm International Water Institute. Stockholm, Sweden, pp. 13-24.

Aycicek, H., Oguz, U., Karci, K. 2006. Determination of total aerobic and indicator bacteria on some raw eaten vegetables from wholesalers in Ankara, Turkey. Int. J. Hyg. Environ. Health, 209, pp. 197-201.

Bae, S., Wuertz, S. 2009. Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with propidium monoazide. Appl. Environ. Microbiol., 75, pp. 2940-2944.

Baggi, F., Demarta, A., Peduzzi, R. 2001. Persistence of viral pathogens and bacteriophages during sewage treatment: lack of correlation with indicator bacteria. Res. Microbiol., 152, pp. 743-751.

Bahri, A., Drechsel, P., Brissaud, F. 2008. Water reuse in Africa. Opportunities and challenges. First African Water Week. Tunisia. March, 26-28.

Baker, K.H., Hegarty, J.P. 2001. Presence of *Helicobacter pylori* in drinking water is associated with clinical infection. Scand. J. Infect. Dis., 33, pp. 744-746.

Beauté, J., Zucs, P., de Jong, B. 2013. Legionnaires' disease in Europe, 2009-2010. Eurosurveill., 18, pp. 1-7. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20417

Benin, A.L., Benson, R.F., Besser, R.E. 2002. Trends in legionnaires disease, 1980-1998: declining mortality and new patterns of diagnosis. Clin. Infect. Dis., 35, pp. 1039-1046.

Benson, R.F., Fields, B.S. 1998. Classification of the genus *Legionella*. Semin. Respir. Infect., 13, pp. 90-99.

Bentsink, L., Leone, G.O., van Beckhoven, J.R., van Schijndel, H.B., van Gemen, B., van der Wolf, J.M. 2002. Amplification of RNA by NASBA allows direct detection of viable cells of *Ralstonia solanacearum* in potato. J. Appl. Microbiol. 93, pp. 647–655.

Bessetti, J. 2007. An introduction to PCR inhibitor. Profiles in DNA, Promega Corporation, pp. 9-10.

Bishop, P.L. 2007. The role of biofilms in water reclamation and reuse. Water Sci. Technol., 55, pp.19-26.

Bitton, Gabriel. 2005. Wastewater Microbiology. John Wiley & Sons, Inc., Hoboken, New Jersey, US.

Blatt, S.P., Parkinson, M.D., Pace, E., Hoffman, P., Dolan, D., Lauderdale, P., Zajac, R.A., Melcher, G.P. 1993. Nosocomial Legionnaires' disease: aspiration as a primary mode of disease acquisition. Am. J. Med., 95, pp. 16-22.

Bolong, N., Ismail, A.F., Salim, M.R., Matsuura, T. 2009. A review of the effects of emerging contaminants in wastewater and options for their removal. Desalination, 239, pp. 229–246.

Boyer, M., Combrisson, J. 2013. Analytical opportunities of quantitative polymerase chain reaction in dairy microbiology. Int. Dairy J., 30, pp. 45-52.

Brenner, A. 2012. Limitations and challenges of wastewater reuse in Israel. Clean Soil and Safe Water. NATO Science for Peace and Security Series C: Environmental Security, pp. 3-9.

Brescia, C.C., Griffin, S.M., Ware, M.W., Varughese, E.A., Egorov, A.I., Villegas, E.N. 2009. *Cryptosporidium* propidium monoazide-PCR, a molecular biology-based technique for genotyping of viable *Cryptosporidium* oocysts. Appl. Environ. Microbiol., 75, pp. 6856-6863.

Burke, V., Robinson, J., Gracey, M., Petersen, D., Patridge, K. 1984. Isolation of *Aeromonas hydrofila* from a metropolitan water supply: seasonal correlation with clinical isolates. Appl. Environm. Microbiol., 48, pp. 361-366.

Bustin, S.A., Benes, V., Garson, J.A., Hallemans, J., Huggeh, J., Kubista, M., Mueller, R., Nolan, T., Pfattl, M.W., Shipley, G.L., Vandesompele, J., Willwer, C.T. 2009. The MIQE guidelines: minimum information for publication of quantitative real time PCR experiments. Clin. Chem., 55, pp. 611-622.

Bustin, S.A. 2010. Why the need for qPCR publication guidelines?-the case for MIQE. methods, 50, pp. 217-226.

Calderón Preciado, D., Matamoros, V., Savé, R., Muñoz, P., Biel., C., Bayona, J.M. 2013. Uptake of microcontaminants by crops irrigated with reclaimed water and groundwater under real field greenhouse conditions. Environ. Sci. Pollut. Res., 20, pp. 3629-3638.

Caliman, F.A., Gavrilescu, M. 2009. Pharmaceuticals, personal care products and endocrine disrupting agents in the environment – a review. Clean, 37 (4-5), pp. 277-303.

Cano Portero, R., Martín Mesonero, C., Pelaz Antolín, C. 2010. Brotes de legionelosis notificados a la Red Nacional de Vigilancia Epidemiológica. Años 1999 a 2009. Boletín Epidemiológico Semanal, 18, pp. 161-164.

Cao-Hoang, L., Marechal, P.A., Le-Thanh, M., Gervais, P., Wache, Y. 2008. Fluorescent probes to evaluate the physiological state and activity of microbial biocatalysts: a guide for prokaryotic and eukaryotic investigation. Biotechnol. J., 3, pp. 890-903.

Carratalà, J., García-Vidal, C. 2010. An update in *Legionella*. Curr. Opin. Infect. Dis., 23, pp. 152-157.

Castro, G.A. 1996. Helminths: structure, classification, growth, and development. In: Medical Microbiology (Chapter 86). 4th edition. Baron, S., (Ed.). University of Texas Medical Branch at Galveston, Galveston, Texas, US.

Cawthorn, D.M., Witthuhn, R.C. 2008. Selective PCR detection of viable *Enterobacter sakazakii* cells utilizing propidium monoazide or ethidium bromide monoazide. Appl. Environ. Microbiol., 105, pp. 1178–1185.

CEDEX. 2008. Realización de una base de datos sobre los sistemas de reutilización de aguas depuradas en España, Madrid. In: Melgarejo, J. 2009. Efectos ambientales y económicos de la reutilización del agua en España. Clm. Economía, 15, pp. 245-270.

Cenciarini-Borde, C., Courtois, S., La Scola, B. 2009. Nucleic acids as viability markers for bacteria detection using molecular tools. Future Microbiol., 4, pp. 45-64.

Chatzisymean, E., Droumpali, A., Montzavinos, D., Venieri, D. 2011. Disinfection of water and wastewater by UV-A and UV-C irradiation: application of real-time PCR method. Photochem. Photobiol. Sci., 10, pp. 389-395.

Chetta, M, Bafunno, V., Grillo, R., Mele, A., Lo Perfido, P., Notarnicola, M., Cellini, F., Chifarelli, R.A. 2012. SYBR green real time-polymerase chain reaction as a rapid and alternative assay for the efficient identification of all existing *Escherichia coli* biotypes approved directly in wastewater samples. Biotechnol. Prog., 28, pp. 1106-1113.

Cirelli, G.L., Consoli, S., Licciardello, F., Aiello, R., Giuffrida, F., Leonardi, C. 2012. Treated municipal wastewater reuse in vegetable production. Agr. Water Manage., 104, pp. 163-170.

Codony, F., Pérez, L.M., Morató, J., Domingues Gual, M.C. 2009. Utilidad de las técnicas de biología molecular para el control analítico de la calidad microbiológica de las aguas. Tecnología del agua, 313, pp. 66-69.

Codony, F., Pérez, L.M., Adrados, B., Agustí, G., Fittipaldi, M., Morató, J. 2012. Amoeba-related health risk in drinking water systems: could monitoring of amoebae be a complementary approach to current quality control strategies?. Future Microbiol., 7, pp. 25-31.

Costán-Longares, A., Montemayor, M., Payán, A., Méndez, J., Jofre, J., Mujeriego, R., Lucena, F. 2008. Microbial indicators and pathogens: removal, relationships and predictive capabilities in water reclamation facilities. Water Res., 42, pp. 4439-4448.

Costerton, J.W. 1995. Overview of microbial biofilms. J. Ind. Microbiol., 15, pp. 137-140.

Danese, P.N., Pratt, L.A., Kolter, R. 2000. Exopolysaccharide production is required for the development of *E. coli* K-12 biofilm architecture. J. Bacteriol., 182, pp. 3593-3596.

Daughton, C.G. 2004. Non-regulated water contaminants: emerging research. Environ. Impact Assess. Rev., 24, pp., 711-732.

Declerck, P. 2010. Biofilms: the environmental playground of *Legionella pneumophila*. Environ. Microbiol., 12, pp. 557-566.

De Koning, J., Bixio, D., Karabelas, A., Salgot, M., Schäfer, A. 2008. Characterisation and assessment of water treatment technologies for reuse. Desalination, 218, pp. 92-104.

Delgado Viscogliosi, P., Solignac, L., Delattre, J.M. 2009. Viability PCR, a culture independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. Appl. Environ. Microbiol., 75, pp. 3502- -3512.

Diereden, B.M.W. 2008. *Legionella* spp. and Legionnaires' disease. J. Infection, 56, pp. 1-12.

Dong, Y., Kim, J., Lewis, G.D. 2010. Evaluation of methodology for detection of human adenoviruses in wastewater, drinking water, stream water and recreational waters. J. Appl. Microbiol., 108, pp. 800-809.

Engel, H.W.B., Berwald, L.G., Havelaar, A.H. 1980. The occurrence of *Mycobacterium kansasii* in tap water. Tubercle, 61, pp. 21-26.

EPRI. 2003. Use of degraded water sources as cooling water in power plants, 1005359. California Energy Commission. Public Interest Energy Research Program, Sacramento, California, US. In: Hsieh, M.K., Li, H., Chien, S.H., Monnell, J.D., Chowdhury, I., Dzombak, D.A., Vidic, R.D. 2010. Corrosion control when using secondary treated municipal wastewater as alternative makeup water for cooling tower systems. Water Environ. Res., 82, pp. 2346-2356.

EPSAR (Entidad Pública de Saneamiento de Aguas Residuales de la Comunidad Valenciana). 2012. Reutilización de aguas depuradas en la comunidad valenciana. Oral presentation. IX Congreso Internacional AEDyR. November, 13-15. Madrid, Spain.

Exall, K., Marsalek, J., Schaefer, K. 2006. Water reuse in Canada: opportunities and challenges. Integrated Urban Water Resources Management. Springer, Netherlands, pp. 253-262.

Farah, A.N., Batarseh, M.I. 2008. Agricultural reuse of reclaimed water and uptake of organic compounds: pilot study at Mutah University wastewater treatment plant, Jordan. Chemosphere, 72, pp. 1203-1214.

Fatta, D., Anayiotou, S. 2007. MEDAWARE project for wastewater reuse in the Mediterranean countries: An innovative compact biological wastewater treatment system for promoting wastewater reclamation in Cyprus. Desalination, 211, pp. 34-47.

Fatta Kassinos, D., Kalavrouziotis, I.K., Koukoulakis, P.H., Vazquez, M.I. 2011. The risks associated with wastewater reuse and xenobiotics in the agroecological environment. Sci. Total Environ., 409, pp. 3555-3563.

Fenton, S.E. 2006. Endocrine-disrupting compounds and mammary gland development: Early Exposure and Later Life Consequences. Endrocrinology, 147, pp. s18 – s24.

Ferrer, A., Nguyen-Viet, H., Zinsstag, J. 2012. Quantification of diarrhea risk related to wastewater contact in Thailand. Ecohealth, 9, pp. 49-59.

Fittipaldi, M., Rodriguez, N. J., Codony, F., Adrados, B., Peñuela, G., Morató, J. 2010. Discrimination of infectious bacteriophage T4 virus by propidium monoazide real-time PCR. J. Virol. Methods, 168, pp. 228-232.

Fittipaldi, M., Pino Rodriguez, N.J., Adrados, B., Agustí, G., Peñuela, G., Morató, J. 2011. Discrimination of viable *Acanthamoeba castellani* trophozoites and cysts by propidium monoazide real-time polymerase chain reaction. J. Eukaryot. Microbiol., 58, pp. 359-364.

Flemming, H.C., Wingender, J. 2010. The biofilm matrix. Nat. Rev. Microbiol., 8, pp. 623-633.

Fux, C.A., Costerton, J.W., Stewart, P.S., Stoodley, P. 2005. Survival strategies of infectious biofilms. Trends Microbiol., 13, pp. 34-40.

Gao, J., Wang, H.X., Liu, H.J., Yin, J., Zhao, X. 2013. Effects of reclaimed water iIrrigation upon soil quality. Adv. Mat. Res., 610-613, pp. 3022-3026.

García, A., Goñi, P., Cieloszyk, J., Fernandez, M.T., Calvo-Beguería, L., Rubio, E., Fillat, M.F., Peleato, M.L., Clavel., A. 2013. Identification of free-living amoebae and amoeba-associated bacteria from reservoirs and water treatment plants by molecular techniques. Environ. Sci. Technol., 47, pp 3132–3140.

Gasparetto, M., Pescarin, M., Guariso, G. 2012. *Helicobacter pylori* eradication therapy: current availabilities. ISRN Gastroenterol., 2012, pp. 1-8.

Gerardi, M.H. 2006. Bacteria. In: Gerardi, M.H. 2006. Wastewater bacteria. Jhon Wiley & Sons, Inc. New Jersey, US.

Girones, R., Ferrús, M.A., Alonso, J.L., Rodriguez-Manzano, J., Calgua, B., de Abreu Corrêa, A., Hundesa, A., Carratala, A., Bofill-Mas, S. 2010. Molecular detection of pathogens in water-the pros and cons of molecular techniques. Water Res., 44, pp. 4325-4339.

González, A., Suski, J., Ferrús, M.A. 2010. Rapid and accurate detection of *Arcobacter* contamination in commercial chicken products and wastewater samples by real-time polymerase chain reaction. Foodborne Pathog. Dis., 7, pp. 327-338.

González, S., López-Roldán, R., Cortina, J.L. 2012. Presence and biological effects of emerging contaminants in Llobregat River basin: a review. Environ. Pollut., 161, pp. 83-92.

Green, P.N., Pirrie, R.S. 1993. A laboratory apparatus for the generation and biocide efficacy testing of *Legionella* biofilms. J. Appl. Bacteriol., 74, pp. 388-393.

Guardabassi, L., Petersen, A., Olsen, J.E., Dalsgaard, A. 1998. Antibiotic resistance in *Acinetobacter* spp. isolated from sewers receiving waste effluent from a hospital and pharmaceutical plant. Appl. Environ. Microbiol., 64, pp. 2499-3502.

Haas, C.N., Rose, J.B., Gerba, C.P. (Eds.). 1999. Microbial risk assessment, Wiley, New York. In: Toze, S. 2006. Water reuse and health risks-real vs. perceived. Desalination, 187, pp. 41-51.

Ham, J.H., Yoon, C.G., Jeon, J.H., Kim, H.C. 2007. Feasibility of a constructed wetland and wastewater stabilization pond system as a sewage reclamation system for agricultural reuse in a decentralized rural aerea. Water Sci. Technol., 55, pp. 503-511.

Harwood, V. J., Levine, A.D., Scott, T.M., Chivikula, V., Lukasik, J., Farrah, S.R., Rose, J.B. 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl. Environ. Microbiol., 71, pp. 3163 – 3170.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplifications reactions. Biotechnology, 11, pp. 1026-1030.

Hochstrat, R., Wintgens, T., Melin, T., Jeffrey, P. 2005. Wastewater reclamation and reuse in Europe: a model-based potential estimation. Water Sci. Technol., Water Supply (5-1), pp. 67-75.

Hoofar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., Fach, P. 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J. Clin. Microbiol., 42, pp. 1863-1868.

Hulten, K., Han, S.W., Enroth, H., Klein, P.D., Opekun, A.R., Gilman, R.H., Evans, D.G., Engstrand, L., Graham, D.Y., El-Zaatari, F.A. 1996. *Helicobacter pylori* in the drinking water in Peru. Gastroenterology, 110, pp. 1031-1035.

Innis, M.A., Myambo, K.B., Gelfand, D.H., Brow, M.A. 1988. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. PNAS, 85, pp. 9436-9440.

Jiménez, B., Asano, T. 2008a. Water Reuse: an international survey of current practice, issues and needs. Jiménez, B., Asano, T. (Eds.). 2008. IWA Publishing. London, UK.

Jiménez, B., Asano, T. 2008b. Water reclamation and reuse around the world. Water reuse: an international survey of current practice, issues and needs. Jiménez, B., Asano, T. (Eds.). 2008. IWA Publishing. London, UK. Chapter 1, pp. 3-26.

Jin, X., Yue, S., Wells, K.S., and Singer, V.L. 1994. SYBR Greeen I: a new fluorescent dye optimized for detection of picogram amounts of DNA in gels. Biophys. J., 66, A159.

Jjemba, P.K., Weinrich, L., Cheng, W., Giraldo, E., LeChevallier, M.W., 2010. Guidance document on the microbiological quality and biostability of reclaimed water following storage and distribution. WaterReuse Foundation, Virginia, US.

Johansen, C., Falhot, P., Gram, L. 1997. Enzymatic removal and disinfection of bacterial biofilms. Appl. Environ. Microbiol., 63, pp. 3724-3728.

Juanico, M., Friedler, E. 1999. Wastewater reuse for river recovery in semiarid Israel. Water Sci. Technol., 40, pp. 43-50.

Juanico, M., Salgot, M. 2008. Water reuse in the northern Mediterranean region. Water reuse: an international survey of current practice, issues and needs. Jiménez, B., Asano, T. (Eds.). 2008. IWA Publishing. London, UK. Chapter 3, pp. 48-67.

Kao, P.M., Tung, M.C., Hsu, B.M., Chiu, Y.C., She, C.Y., Shen, S.M., Huang, Y.L., Huang, W.C. 2013. Identification and quantitative detection of *Legionella* spp. in various aquatic environments by real-time PCR assay. Environ. Sci. Pollut. Res., DOI 10.1007/s11356-013-1534-z.

Kaufmann, A.F., McDade, J.E., Patton, C.M., Bennett, J.V., Skaliy, P., Feeley, J.C., Anderson, D.C., Potter, M.E., Newhouse, V.F., Gregg, M.B., Brachman, P.S. 1981. Pontiac fever: isolation of the etiologic agent (*Legionella pneumophila*) and demonstration of its mode of transmission. Am. J. Epidemiol., 114, pp. 337-347.

Keegan, A., Tutuka, C., Monis, P. 2009. Pathogens and indicators in wastewater matrices. Microbiology Australia, 30, pp. 8-11.

Keene, W.E., McAnulty, J.M., Hoesly, F.C., Williams, L.P. Jr., Hedberg, K., Oxman, G.L., Barrett, T.J., Pfaller, M.A., Fleming, D.W. 1994. A swimming-associated outbreak of hemorrhagic colitis caused by *Escherichia coli* O157:H7 and *Shigella sonnei*. N. Engl. J. Med., 331, pp. 579 - 584.

Kohli, A., Frenken, K. 2011. Cooling water for energy generation and its impact on national-level water statistics. AQUASTAT programme of FAO. http://www.fao.org/nr/water/aquastat/catalogues/thermoelectric_cooling_water_20110429.pdf.

Kooij, D., Veenendaal, H.R. 2002. Biofilm formation and multiplication of *Legionella* on synthetic pipe materials in contact with treated water under static and dynamic conditions. In: Nazmiye, O.S., Ayten, K., Aysin, C. 2007. Studies on the efficacy of Chloramine T trihydrate (N-chloro-p-toluene sulfonamide) against planktonic and sessile populations of different *Legionella pneumophila* strains. Int. J. Hyg. Environ. Health, 210, pp. 147-153.

Kool, L.J. 2002. Control of *Legionella* in drinking water systems: impact of monochloramine. In: Marre, R., Kwaik, Y.A., Bartlett, C., Cianciotto, N.P., Fields, B.S., Frosch, M., Hacker, J., Lück, P.S. (Eds.). *Legionella*. ASM Press, Washington, DC, US, pp. 411-418.

Koster, W., Egli, T., Ashbolt, N., Botzenhart, K., Burlion, N., Endo, T., Grimont, P., Guillot, E., Mabilat, C., Newport, L., Niemi, M., Payment, P., Prescott, A., Renaud, P., Rust, A. 2003. Analytical methods for microbiological water quality testing. In: Assessing microbial safety of drinking water: improving approaches and methods. OECD/WHO Drinking Water Quality Series, IWA Publishing, London, UK, pp. 237–295.

Koubar, M., Rodier, M.H., Frère, J. 2013. Involvement of minerals in adherence of *Legionella pneumophila* to surfaces. Curr. Microbiol., 66, pp. 437-442.

Kümmerer, K. 2009. The presence of pharmaceuticals in the environment due to human use – present knowledge and future challenges. J. Environ. Manage., 90, pp. 2354–2366.

Lazarova, V., Asano, T., 2005. Challenges of sustainable irrigation with recycled water, In: Lazarova, V., Bahri, A., (Eds.). 2005. Water reuse for irrigation: agriculture, landscapes, and turf grass. CRC Press, Florida, US, pp. 1-30.

Lazarova, V., Bouwer, H., Bahri, A. 2005. Water quality considerations. In: Lazarova, V., Bahri, A. (eds.). Water Reuse for Irrigation: Agriculture, Landscapes, and Turf Grass. CRC Press, Florida, US, pp. 31-60.

Lemarchand, K., Masson, L., Brousseau, R. 2004. Molecular biology and DNA microarray technology for microbial quality monitoring of water. Crit. Rev. Microbiol., 30, pp. 145-72.

Lievens, B., Brouwer, M., Vanachter, A.C.R.C., Lévesque, C.A., Cammue, B.P.A., Thomma. B.P.H.J. 2005. Quantitative assessment of phytopathogenic fungi in various substrates using a DNA macroarray. Environ. Microbiol., 7, pp. 1698-1710.

Lim, R., Gale, S., Doyle, C., Lesjean, B., Gilbert, M. 2000. Endocrine disrupting compounds in sewage treatment plant (STP) effluent reused in agriculture-is there a concern? In: Proceeding of the first Symposium Water Recycling, Australia, Adelaide, 19-20 October 2000, pp. 23-28.

Mackay, I.M., Mackay, J.F., Nissen, M.D., Sloots, T.P. 2007. Real-time PCR: history and fluorogenic chemistries. In: Mackay, I.M. 2007. Real-time PCR in microbiology. From diagnosis to characterization. Chapter 1. Caister Academic Press, Norfolk, UK.

Mackay, I.M. 2007. Real-time PCR in microbiology. From diagnosis to characterization. Caister Academic Press, Norfolk, UK.

Mackey, W.G., Gribbon, L.T., Barer, M.R., Reid, D.C. 1998. Biofilms in drinking water systems-A possible reservoir for *Helicobacter pylori*. Water Sci.Technol., 38, pp. 181-185.

Madigan, M.T., Martinko, J.M., Parker, J. 1997. Biology of microorganisms. Prentice Hall. Upper Saddle River, New Jersey, US, pp. 156.

Mah, T.F.C., O'Toole, G. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol., 9, pp. 34-39.

Malorny, B., Tassios, P.T., Radström, P., Cook, N., Wagner, M., Hoorfar, J. 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food Microbiol., 83, pp. 39-48.

Marecos do Monte, M.H. 2007. Water reuse in Europe. Official Publication of the European Water Association (EWA), pp. 1-18.

Mastro, T.D., Fields, B.S., Breiman, R.F., Campbell, J., Plikaytis, B.D., Spika, J.S. 1991. Nosocomial Legionnaires' disease and use of medication nebulizers. J. Infect. Dis., 163, pp. 667-671.

McCabe, W.L., Smith, J.C. 2002. Operaciones Unitarias en Ingeniería Química. McGraw-Hill Interamericana Editors, S.A., C.V. Mexico.

McCann, B. 2012. Wastewater reuse brings life back to Spain's Segura river. Water 21, 14.4, pp. 28-33.

McCartney, H. A., Foster, S. J., Fraaije, B. A., and Ward, E. 2003. Molecular diagnostics for fungal plant pathogens. Pest Manag. Sci., 59, pp. 129-142.

Mekata, T., Sudhakaran, R, Itami, T. 2012. Development and evaluation of real-time loop-mediated isothermal amplification methods for the rapid detection of penaeid viruses. Bull. Fish. Res. Agen., 35, pp. 39-50.

Melgarejo, J. 2009. Efectos ambientales y económicos de la reutilización del agua en España. Clm. Economía, 15, pp. 245-270.

Metcalf & Eddy Inc., Tchobanoglous, G. 1991. Wastewater Engineering, Treatment, Disposal and Reuse. McGraw-Hill International. Singapore.

Metcalf & Eddy Inc. an AECOM company, Asano, T., Burton, F., Leverenz, H., Tsuchihashi, R., Tchobanoglous, G. 2007. Water reuse: issue, technologies, and applications. McGraw Hill Professional. New York, US.

Moellering Jr, R.C. 1991. The enterococcus: a classic example of the impact of antimicrobial resistance on therapeutic options. J. Antimicrob. Chemother., 28, pp. 1-12.

Momba, M.N.B., Binda, M.A. 2002. Combining chlorination and chloramination processes for the inhibition of biofilm formation in drinking surface water system models. J. Appl. Microbiol., 92, pp. 641-648.

Morrison, T.B., Weis, J.J., Wittwer, C.T. 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques, 24, pp. 954–962.

Mujeriego, R. 1990. Manual práctico de riego con agua residual municipal regenerada. Edicions de la Universitat Politècnica de Catalunya. Barcelona, Spain.

Mujeriego, R. 1998. Evolución y perspectiva de la reutilización de aguas en España, Edicions de la Universitat Politècnica de Catalunya. Barcelona, Spain.

Mujeriego, R. 2006. La reutilización planificada del agua para regadío. La reutilización planificada de agua: aspectos reglamentarios, sanitarios, técnicos y de gestión. XI Congreso Nacional de Regantes, April 15-20, Palma de Mallorca, Spain.

Mujeriego, R. 2007. La reutilización, la regulación, y la desalación en la gestión integrada del agua. In La sequía en España. Directrices para minimizar su impacto. Chapter 7. Ministerio de Medioambiente. España.

Mujeriego, R., Asano, T. 1999. The role of advanced treatment in wastewater reclamation and reuse. Water Sci. Technol., 40, pp. 1-9.

Mullis, K.B. 1990. The unusual origin of the polymerase chain reaction. Sci. Am., 262, pp. 56-61.

Nakada, N., Tanishima, T., Shinohara, H., Kiri, K., Takada, H. 2006. Pharmaceutical chemicals and endocrine disrupters in municipal wastewater in Tokyo and their removal during activated sludge treatment. Water Res., 40, pp. 3297-3303.

Nataro, J.P., J.B. Kaper. 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev., 11, pp. 142–201.

National Research Council. 1999. Identifying future drinking water contaminants. The National Academies Press. Washington D.C., US, pp. 189.

Nayak, A.K., Rose, J.B. 2007. Detection of *Helicobacter pylori* in sewage and water using a new quantitative PCR method with SYBR® green. J. Appl. Microbiol., 103, pp. 1931-1941.

Nkuipou-Kenfack, E., Engel, H., Fakih, S., Nocker, A. 2013. Improving efficiency of viability-PCR for selective detection of live cells. J. Microbiol. Methods, 93, pp. 20-24.

Nocker, A., Camper, A.K. 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl. Environ. Microbiol. 72, pp. 1997–2004.

Nocker, A., Cheung, C.Y., Camper, A.K., 2006. Comparison of propidium monoazide and ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Methods, 67, pp. 310–320.

Nocker, A., Burr, M., Camper, A.K. 2009. Synthesis document on molecular techniques for the drinking water industry. Chapter 2, pp. 5. Water Research Foundation. US.

Nogva, H.K., Dromtorp, S.M., Nissen, H., Rudi, K., 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5′-nuclease PCR. BioTechniques, 810, pp. 812–813.

Novak, J.S., Juneja, V.K. 2001. Detection of heat injury in *Listeria monocytogenes* Scott A. J. Food Prot., 64, pp. 1739–1743.

Ogoshi, M. Suzuki, Y., Asano, T. 2001. Water reuse in Japan. Water Sci. Technol., 43, pp. 17-23.

Oliver, J.D. 2005. The viable but nonculturable state in bacteria. J. Microbiol., 43 (S), pp. 93-100.

Opel, K.L., Chung, D., McCord, B.R. 2010. A Study of PCR Inhibition Mechanisms Using Real Time PCR. J.Forensic Sci., 55, 25-33.

Ortega de Miguel, E., Iglesias Esteban, R. 2007. La reutilización de las aguas depuradas en España. Normativa y tecnologías más utilizadas. SMALLWAT, oral communication, Sevilla, Spain.

Ortega de Miguel, E., Iglesias Esteban, R. 2008. Present and future of wastewater reuse in Spain. Desalination, 218, pp. 105-119.

O'Toole, G., Kaplan, H.B., Kolter, R. 2000. Biofilm formation as microbial development. Annu. Rev. Microbiol., 54, pp. 49-79.

Peasey, A., Blumenthal, U., Mara, D., Ruiz-Palacio, G. 2000. A review of policy and standards for wastewater reuse in agriculture: a Latin American perspective. WELL Study no. 68, Part II, London, UK.

Pedrero, F., Allende, A., Gil, M.I., Alarcón, J.J. 2012. Soil chemical properties, leaf mineral status and crop production in a lemon tree orchard irrigated with two types of wastewater. Agr. Water Manage., 109, pp. 54-60.

Peist, R., Honsel, D., Twieling, G., Löffert, D. 2001. PCR inhibitors in plant DNA preparations. Qiagen News, 3, pp. 7-9.

Petrovic, M., Barceló, D. 2012. Inputs of pharmaceuticals and endocrine disrupting compounds in the Llobregat river basin. Hdb. Env. Chem., 21, pp. 151-165.

Rajal, V.B., McSwain, B.S., Thompson, D.E., Leutenegger, C.M., Kildare, B.J., Wuerts, S. 2007. Validation of hollow fiber ultrafiltration and real time PCR using bacteriophage PP7 as surrogate for the quantification of viruses from water samples. Water Res., 41, pp. 1411-1422.

Ramakers, C., Ruijter, J.M., Lekanne Deprez, R.H., Moorman, A.F.M. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett., 339, pp. 62-66.

Rasmussen, R. 2001. Quantification on the LightCycler. In: Meuer, S., Wittwer, C., Nakagawara, K. (Eds.). 2001. Rapid cycle real-time PCR - methods and applications. Springer Press, Heidelberg, pp. 21-34.

Rawsthorne, H., Dock, C. N., Jaykus, L. A. 2009. PCR-based method using propidium monoazide to distinguish viable from non-viable *Bacillus subtilis* spores. Appl. Environ. Microbiol., 75, pp. 2936--2939.

Rengasamy, P. 2010. Soil processes affecting cop production in salt-affected soils. Funct. Plant Biol., 37, pp. 613-620.

Ririe, K.M., Rasmussen, R.P., Wittwer, C.T. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem., 245, pp. 154-160.

Rodriguez Lázaro, D., Hernández, M. 2013. Real-time PCR in food science: introduction. Curr. Issues Mol. Biol., 15, pp. 25-38.

Rogers, P.P., Llamas, M.R., Cortina, L. 2006. Water crisis: mith or reality?. Taylor & Francis plc., London, UK.

Rompré, A., Servais, P., Baudart, J., de Roubin, M.R., Laurent, P. 2002. Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J. Microbiol. Methods, 49, pp. 31-54.

Rosmini, F., Castellani-Pastoris, M., Mazzotti, M.F., Forastiere, F., Gavazzoni, A., Greco, D., Ruckdeschel, G., Tartagni, E., Zampieri, A., Baine, W.B. 1984. Febrile illness in successive cohorts of tourists at a hotel on the Italian Adriatic coast: evidence for a persistent focus of *Legionella* infection. Am. J. Epidemiol., 119, pp. 124-134.

Rossen, L., Norskov, P., Holmstrom, K., Rasmussen, O.F. 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int. J. Food Microbiol., 17, pp. 37-45.

Royal Decree-Law 11/1995 of urban wastewater treatment. Official Bulletin of State, BOE, 312.

Royal Decree 2210/1995 which created the National Epidemiologic Surveillance Network. Official Bulletin of state, BOE, 21.

Royal Decree-Law 1/2001 of Water Act. Official Bulletin of State, BOE, 176.

Royal Decree-Law 1620/2007 of treated wastewater reuse. 2007. Official Bulletin of State, BOE, 294.

Salgot, M., Huertas, E., Weber, S., Dott, W., Hollender, J. 2006. Wastewater reuse and risk: definition of key objectives. Desalination, 187, pp. 29-40.

Sanchez, G., Elizaquivel, P., Aznar, R. 2012. Discrimination of infectious hepatitis A viruses by propidium monoazide real-time RT-PCR. Food Environ. Virol., 4, pp. 21-25.

Sanz, J.L., Köchling, T. 2007. Molecular biology techniques used in wastewater treatment: an overview. Process Biochem., 42, pp.119-133.

Savichtcheva, O., Okabe, S. 2006. Alternative indicators of fecal pollution: relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. Water Res., 40, pp. 2463-2476.

Savichtcheva, O., Okayama N., Okabe S., 2007. Relationships between *Bacteroides* 16S rRNA genetic markers and presence of bacterial enteric pathogens and conventional fecal indicators. Water Res., 41, pp. 3615-3628.

Savichtcheva, O., Okabe, S., 2009. Qualitative and quantitative estimation of host-specific fecal pollution using *Bacteroides-Prevotella* 16S rRNA genetic markers by T-RFLP and real-time PCR analyses. Water Sci. Technol., 59, pp. 1831-1840.

Schrader, C., Shielke, A., Ellerbroek, L, Johne, R. 2012. PCR inhibitors occurrence, properties and removal. J. Appl. Microbiol., 113, pp. 1014-1026.

Shannon, K.E., Lee, D.Y., Trevors, J.T., Beaudette, L.A. 2007. Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. Sci. Total Environ., 382, pp. 121-129.

Sheikh, B., Jaques, R.S., Cort, R.P. 1987. Reuse of tertiary municipal wastewater effluent for irrigation of raw-eaten food crops: a 5 year study. Desalination, 67, pp. 245-254.

Shenker, M., Harush, D., Ben-Ari, J., Chefetz, B. 2011. Uptake of carbamazepine by cucumber plats- a case study related to irrigation with reclaimed wastewater. Chemosphere, 82, pp. 905-910.

Sheridan, G.E., Masters, C.I., Shallcross, J.A., MacKey, B.M. 1998. Detection of mRNA by reverse transcription-PCR as an indicator of viability in *Escherichia coli* cells. Appl. Environ. Microbiol. 64, pp. 1313–1318.

Shi, H., Xu, W., Trinh, Q., Luo, Y., Liang, Z., Li, Y., Huang, K. 2012. Establishment of a viable cell detection system for microorganisms in wine based on ethidium monoazide and quantitative PCR. Food Control, 27, pp. 81-86.

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Takade, A., Yoshida, S. 2007. Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria. Microbiol. Immunol., 51, pp. 763-775.

Sood, S., Malhotra, M., Das, B.K., Kapil, A. 2008. Enterococcal infections & antimicrobial resistance. Indian J. Med. Res., 128, pp. 111-21.

Souaze, F., Ntodou-Thome, A., Tran, C.Y., Rostene, W., Forgez, P. 1996. Quantitative RT-PCR: limits and accuracy. BioTechniques, 21, pp. 280-285.

Spano, G., Beneduce, L., Terzi, V., Stamca, A.M., Massa, S. 2005. Real-time PCR for the detection of *Escherichia coli* O157:H7 in dairy and cattle wastewater. Lett. Appl. Microbiol., 40, pp. 164-171.

Stenco. 2007. Tratamiento de aguas. TINGS. Servicios de comunicación. Barcelona, Spain.

Stout, J.E., Yu, L.V. 1997. Legionellosis. New Engl. J. Med., 337, pp. 682-687.

Stratton, H., Matthews, B. 2009. Pathogens in recycled water: are they measurable?. Microbiol. Australia, 30, pp. 12-15.

Sui, Q., Huang, J., Deng, S., Yu, G., Fan, Q. 2010. Occurrence and removal of pharmaceuticals, caffeine and DEET in wastewater treatment plants of Beijing, China. Water. Res., 44 (2), pp. 417–426.

Tchobanoglous, G., Burton, F.L., Stensel, H.D. 2003. Wastewater engineering: treatment and reuse. Fourth Edition. Metcalf & Eddy, Inc. MacGraw-Hill, New York, US.

Thomas, V., Bouchez, T., Nicolas, V., Robert, S., Loret, J.F., Lévi Y. 2004. Amoebae in domestic water systems: resistance to disinfection treatments and implication in *Legionella* persistence. J. Appl. Microbiol., 97, pp. 950-963.

Thomas, V., McDonnell, G., Denyer, S.P., Maillard, J.Y. 2010. Free-living amoebae and their intracellular pathogenic microorganisms: risks for water quality. FEMS Microbiol. Rev. 34, pp. 231–259.

Toze, S. 2006. Water reuse and health risks-real vs. perceived. Desalination, 187, pp. 41-51.

USEPA. 2004. Guidelines for water reuse, EPA-625/R-04-108, U.S. Environmental Protection Agency and U.S. Agency for International Development, Washington, DC, US.

Valasek, M.A., Repa, J.J. 2005. The power of real-time PCR. Adv. Physiol Educ., 29, pp. 151-159.

Varma, M., Field, R., Stinson, M., Rukovets, B., Wymer, L., Haugland, R. 2009. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. Water Res., 43, pp. 4790–4801.

Vergis, E. N., Akbas, E., Yu, V.L. 2000. Seminars in respiratory and critical care medicine, 21, pp. 295-304.

Vigneswaran, S., Sundaravadivel, M. 2004. Recycle and reuse of domestic wastewater. In: Vigneswaran, S., Sundaravadivel, M. (Eds). 2004. Wastewater recycle, reuse, and reclamation - recycle and reuse of domestic wastewater. Encyclopedia of Life Support Systems (EOLSS). EOLSS Publishers, Oxford, UK.

Westcot, D.W. and Ayers, R.S. 1985. Irrigation water quality criteria. In: Irrigation with reclaimed municipal wastewater – A guidance manual. G.S. Pettygrove, G.S., T. Asano, T. (Eds). 1985. Lewis Publishers, Inc., Chelsea, US.

Wexler, H. M. 2007. *Bacteroides*: the good, the bad, and the nitty-gritty. Clin. Microbiol. Rev., 20, pp. 593-621.

Williams, R.B. 1982. Wastewater reuse - an assessment of the potential and technology. In: Water Reuse. E.J. Middlebrooks, (Ed.). Ann Arbor Science Publishers, Inc., Michigan, US.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol., 63, pp. 3741-3751.

Wingender, J., Flemming, H.C. 2011. Biofilms in drinking water and their role as reservoir for pathogens. Int. J. Hyg. Envir. Health, 214, pp. 417-423.

Wong, M.L., Medrano, J.F. 2005. Real-time PCR for mRNA quantitation. BioTechniques, 39, pp. 75-85.

World Health Organization. 1989. Health guidelines for the use of wastewater in agriculture and aquaculture, report of a WHO scientific group. Technical Report Series 778, World Health Organization, Geneva, Switzerland.

World Health Organization, 2008. WHO: guidelines for drinking-water quality: microbial fact sheets (Chapter 11). World Health Organization, Geneva, Switzerland.

Wright, J.B., Ruseska, I., Consterton, J.W. 1991. Decreased biocide susceptibility of adherent *Legionella pneumophila*. J. Appl. Bacteriology, 71, pp. 531-538.

Yang, X., Flowers, R.C., Weinberg, H.S., Singer, P.C. 2011. Occurrence and removal of pharmaceuticals and personal care products (PPCPs) in an advanced wastewater reclamation plant. Water. Res., 45, pp. 5218-5228.

Yi, L., Jiao, W., Chen, X., Chen, W. 2011. An overview of reclaimed water reuse in China. J. Environm. Sciences, 23, pp. 1585–1593.

Yu, V.L. 1993. Could aspiration be the major mode of transmission for *Legionella*?. Am. J. Med., 95, pp. 13-15.

Chapter 3

Quantitative PCR using SYBR Green for detection of *Legionella pneumophila* in water samples*

This chapter is dedicated to the development and preliminary evaluation of a Polymerase Chain Reaction (PCR) assay using SYBR Green in an attempt to provide a simple screening method for *Legionella pneumophila* in water systems samples. The inexpensive, sensitive and rapid real-time PCR (qPCR) based in SYBR Green method is of interest in monitoring *Legionella pneumophila* contamination, especially in environmental samples, and should be economical for large-scale routine tests.

A total of 50 samples from cooling towers and hot tap-water systems were analysed by DNA amplification using two pairs of primers targeting the *mip* and *dot* genes. *Legionella pneumophila* serogroup 1 (NCTC12821) was used as a reference strain and to evaluate real-time PCR performance. The assays were successful with both primer sets; good and similar amplification efficiencies were achieved. In addition, high sensitivity was obtained; the method proved to allow for the detection of fewer than 10 gene copies per reaction. Results of qPCR were compared to conventional analysis based on culture. Although no strong correlation was observed between both methods and consequently qPCR could not substitute for the reference method, it represents a powerful screening tool.

*An early version of this chapter was published as:

Fittipaldi, M., Codony, F., Morató, J. 2010. Comparison of conventional culture and real-time quantitative PCR using SYBR Green for detection of *Legionella pneumophila* in water samples. Water SA, 36, pp. 417-424.

3.1. Introduction

Legionellae are ubiquitous in the natural environment being present in soils and aquatic ecosystems (Casati et al., 2009; Fliermans et al., 1981; Fliermans, 1996; Joseph and Ricketts, 2010;). *Legionella* sometimes survives as an intracellular parasite of amoebae and ciliates (Codony et al., 2012; Greub and Raoult, 2004; Ratcliff et al., 1998). They are present in process facilities water systems such as cooling towers, water networks in hospitals, industrial or residential buildings and hydrothermal devices among others. As a consequence of appropriate temperature and lack of or poor disinfection, levels of legionellae may be important in those systems.

Respiratory infection by *Legionella pneumophila* is mainly attributed to contaminated water aerosols inhalation (Grabow, 1991; Anonymous, 1994) produced by systems such as cooling towers (Lin et al., 2009; Rosmini et al., 1984), showers (Mastro et al., 1991), and nebulizers (Blatt et al., 1993). Aspiration of contaminated water aerosols has also been proposed as a possible mechanism of transmission (Yu, 1993; Steinert et al., 1997).

Legionellosis is generally considered a preventable illness because controlling or eliminating the bacterium in certain reservoirs will (in theory) prevent disease. This fact has resulted in a number of guidelines and control strategies aimed at reducing the risk of legionellosis in building water systems (Diereden, 2008). Risk assessment and management approach is taken as well by the American Society of Heating, Refrigerating and Air conditioning Engineers (ASHRAE) and the U.K. Health and Safety Executive (Mascone, 2008). *Legionella* levels in water are controlled routinely by culture on a selective medium like Buffered Charcoal Yeast Extract (BCYE) or Glycine Vancomycin Polymyxin Cycloheximide agar (GVPC) but its slow growth is a serious drawback, given that it requires at least 10 days to obtain results. Standard culture methods are time consuming and special reagents, culture media, and a high degree of technical skill are required in their application (Bartie et al., 2001) because it is difficult to isolate *Legionella* in waters in the presence of high concentrations of other heterotrophic bacteria. However until now, they have been proven as useful tools. Nevertheless, in the Hazard Analysis Critical Control Points (HACCP) in environments or during outbreak investigation this delay is a serious drawback.

In the last years PCR methods have become the alternative to detect the presence of *Legionella* in a few hours through amplification of specific DNA sequences (Chen et al., 2012; Murdoch et al., 2013; Wójcik-Fatla et al., 2012). The evolution of conventional PCR to real-time PCR has improved even more this situation. The procedure follows the general principle of PCR, but its key feature is that the amplified DNA can be quantified as it accumulates in the reaction in real time after each amplification cycle. Two common chemistries for the quantification are the use of fluorescent dyes that intercalate to double-stranded DNA, and labeled DNA oligonucleotide probes that release fluorescence each time that a new DNA copy is generated.

Multiple *Legionella* detection assays have been published using different target genes (Diederen et al., 2008; Yañez et al., 2005), probes chemistries (Behets et al., 2007; Joly et al., 2006) and real-time thermal cyclers (StØlhaug and Bergh, 2006; Yaradou et al., 2007), but until the moment this work was performed, little was known about the feasibility of a procedure based on SYBR Green. However, there were some reports which demonstrated that SYBR Green could be used for detection of pathogenic microorganisms (Somogyvari et al., 2007; Kares et al., 2004; O´Mahony and Hill, 2002). SYBR Green is a non-specific dye which binds to any double-stranded DNA. Therefore, it will not only bind to the PCR product but to non-specific products that have been amplified as well as primer dimers. Due to this non-specificity, SYBR Green requires more time for optimization than protocols based in primers and probes. Nevertheless, when SYBR Green is used as the fluorescent dye, a subsequent melting curve analysis of PCR products generates a specific profile (depicting the fluorescence change rate over time as a function of temperature for each sample), that can be used to determine the success of the PCR reaction (O´Mahony and Hill, 2002). Furthermore, the use of master mixes based on SYBR Green chemistries is simple, fast and inexpensive.

The objective of this study was to develop a simple, screening qPCR assay for *Legionella pneumophila* in water samples, using SYBR Green-based detection method on two pairs of primers targeting the *mip* and *dot* genes. These primers were previously published as part of a set of primers-probes (Hayden et al., 2001; Yañez et al., 2005). Results of real-time PCR were compared with conventional analysis based on culture.

3.2. Materials and Methods

3.2.1. Water samples and culture

A total of 50 samples were analysed. All samples were from urban areas around Barcelona city (North-East Spain), with two different origins: cooling tower (20 samples) or hot tap water from big buildings (30 samples). Water samples were collected in Pyrex bottles and the quantification of *Legionella pneumophila* by culture was performed according to international standard ISO 11731:1998 (ISO) using culture media (GVPC) and reagents from OXOID. The quantification limit was 5.10^1 colony forming units per liter (CFU/L).

3.2.2. Sample concentration and DNA extraction

A liter of water for each sample was concentrated by membrane filtration using a nylon membrane (0.45 µm pore diameter, Millipore). Cells were re-suspended in 10 mL of saline solution by vigorous vortexing of the filter for 60 seconds with 5 glass beads (5 mm diameter) and sonication for 3 minutes in an ultrasound water bath (Selecta 40 W power, 40 kHz ultrasound frequency). The cell suspension was again concentrated to 2 mL and then to 150 µL by centrifugation (14000 rpm, 5 minutes) using a MiniSpin centrifuge (Eppendorf, Hamburg, Germany). DNA was extracted with DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

3.2.3. Quantitative PCR optimization

The procedure was set up using previously reported primers targeting *dot* (Yañez et al., 2005) and *mip* genes (Hayden et al., 2001). Three key points of qPCR reactions were considered for method adaptation and optimization: annealing temperature and primers and $MgCl_2$ concentrations. Optimization of each parameter was performed by modifying one of them while maintaining the rest fixed to a given value (One-factor-at-a-time, OFAT). The sequence used was the same described previously (temperature, primer concentration, and $MgCl_2$ concentration). At each step, the optimum value of each parameter was selected using as criteria the highest annealing temperature and the lowest threshold cycle (Ct), because under those conditions specificity and sensitivity are maximized while dimerization is minimized. Assays were performed by triplicate in all cases. Real-time PCR conditions evaluated with the selected primers considered annealing temperatures of 55, 56, 57, 58, 59, 60, 61, and 62ºC; primers concentrations of 0.50, 0.75, 1.00, and 1.50 µM and MgCl2 concentrations of 1, 2, 3, 4 and 5 mM.

Evaluation of optimal annealing temperature and primer concentration was carried out using a Quantitect SYBR Green PCR kit (Qiagen, Hilden, Germany), according to manufacturer’s instructions. On the other hand, determination of $MgCl_2$ concentration was performed using a Ligthcycler 1.5 (Roche, Mannheim, Germany) in combination with FastStart DNA masters SYBR Green 1 kit (Roche, Mannheim Germany), according to manufacturer’s instructions. Contribution of $MgCl_2$ in the reaction efficiency, at optimum temperature and primer concentrations, was evaluated using a different mix because this commercial mix does not contain $MgCl_2$ and the level has to be set by the user, while other commercial mix, as Quantitect SYBR Green PCR kit (Qiagen, Hilden, Germany), usually contains $MgCl_2$ in optimum concentration for most purposes.

Primer specificity was re-evaluated in order to ensure their good performance in absence of an internal control. First, a GenBank query was performed. Second, the specificity of our qPCR assay was investigated using DNA extracts of *Legionella pneumophila*, *Legionella* species and non-*Legionella* bacteria (Table 3.1). After culture on their appropriate medium for 24 h, bacterial cells were harvested and suspended in saline solution. After adjusting the concentration of cell suspension at 600 nm, it was 10-fold diluted and a 200 µL aliquot of the dilution related to 10^6 CFU/mL was extracted with the DNeasy Tissue Kit (Qiagen, Hilden, Germany).

Table 3.1. *Legionella* and non-*Legionella* bacteria used in qPCR specificity test.

Bacteria	Source
Legionella pneumophila	NCTC 12821
10 Legionella pneumophila	Environmental isolate according ISO 11731. Confirmed by PCR.
Legionella bozemanii	Environmental isolate according ISO 11731. Confirmed by PCR.
Legionella oakridgensis	NCTC 11531
10 *Legionella* spp.	Environmental isolate according ISO 11731. Confirmed by PCR.
Mycobacterium vaccae	ATCC 14483 T
Helicobacter pylori	Clinical isolate
Bacteroides fragilis	ATCC 51477
E. coli 0157	ATCC 43895
Enterococcus faecalis	ATCC 23655
Staphylococcus aureus	ATCC 12598

3.2.4. *Legionella pneumophila* DNA standard

The DNA used for reaction validation and as reference for *Legionella pneumophila* quantification in water samples, was prepared according to AFNOR XP T90-471 (2006). *Legionella pneumophila* serogroup 1 (NCTC12821) was used as a reference strain and to evaluate real-time PCR performance.

A standard DNA curve was established using a 4-day culture as stock. DNA was obtained with the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The DNA amount was determined by using the Nucleic Acid Quantification analysis method from Ligthcycler 1.5 (Roche, Mannheim, Germany). Briefly, double-stranded DNA (dsDNA) in solution was quantitated by measuring the fluorescent signal in channel 530 (nm). Fluoresce was generated by using SYBR Green quantitation reagent and a dsDNA standard (Maize GMO Standard for NK 603, Fluka Biochemika, Sigma, Madrid, Spain) was

used to create a standard curve. DNA concentration was about 30 (±0.5) ng/µL, as the genome of *Legionella pneumophila* is 4.3 fg (according to AFNOR XP90-471), the number of DNA genomic units (GU) of *Legionella pneumophila* theoretically contained in the extract was evaluated to be 6.05×10^6 GU/µL. Consequently, serial logarithmic dilutions in PCR water, from 10^1 to 10^6 GU per reaction were performed. Amplification efficiency was estimated by means of the slope calculation method from a calibration dilution curve (Rasmussen, 2001). In each case the standard curve was performed by duplicate.

In order to compare GU and CFU values for pure culture, *Legionella pneumophila* serogroup 1 was cultured in *Legionella* selective medium GVPC (Oxoid) for 4 days at 37 ºC. Once the culture was ready, a bacterial suspension was prepared transferring single colonies into sterile saline solution and adjusting the optical density (measured at 600 nm) to 0.2 using a Pharmacia Biotech Novaspec® II spectrophotometer. Serial dilutions were prepared. Each solution was tested by culture and real-time PCR for *mip* and *dot* genes using Ligthcycler 1.5 (Roche, Mannheim, Germany). These assays were performed by duplicate and the corresponding mean values were calculated. Dissociation curves were also recorded after each run.

3.2.5. Quantitative PCR assays

Water sample analysis was performed on a Ligthcycler 1.5 (Roche, Mannheim, Germany). The reaction mixtures for both primers were composed of 9 µL SYBR Green (Quantitect SYBR Green PCR kit, Qiagen, Hilden, Germany), 0.4 U of Uracil-DNA-glycosylase (UDG, New England Biolabs, UK), 9 µL of sample and 0.5 µM of *mip* primers or 0.75 µM of *dot* primers respectively. These concentrations were the results of the qPCR optimization assay.

The experimental protocol consisted of one step of 2 min at 50 ºC to allow UDG to break down the possible contaminating amplicons, one step of 15 min at 95 ºC for Taq polymerase activation, 45 cycles (94 ºC for 15 s, 59 ºC for 30 s, and 72 ºC for 30 s) for DNA amplification and finally a melting temperature ramp from 65 ºC to 95 ºC at 0.1 ºC per second.

For each assay, Ct was determined in order to quantify each DNA product. Quantification was performed by including one or two external standards theoretically containing 4.5×10^4 GU in each set of PCR experiment. The GU number of each sample was determined by comparison to each standard. Each sample was tested by duplicate and the mean value was used for statistical analysis. A negative control (water, PCR grade) was included in all assays. Melting curve analysis was done for all assays to evaluate positive and negative results.

3.2.6. Statistical analyses

In order to compare the equivalence between PCR and conventional microbiology qualitative results ISO/TR 13843:2000 is used as a reference. Statistical analysis to compare culture and PCR results was performed using Microsoft Excel and correlation was established using Pearson's coefficient.

A Chi-square test was used to examine whether two methods (culture and qPCR) were independent or not. This test uses a null hypothesis that it implies that the result achieved by culture and qPCR are independent. Consequently false null hypothesis means that culture and real-time PCR are dependent. In this case Yates' correction is used due to the reduced sample size, given that the use of the Chi-square distribution can introduce some bias in the calculations by making the value of statistical Chi-square greater (Fleiss et al., 2003).

3.3. Results

3.3.1. Quantitative PCR optimization

An adaptation and validation of a qPCR procedure based on SYBR Green with two pairs of primers that targeted the *mip* and *dot* genes was performed. The optimal annealing temperature for the primers was 59 ºC. The optimal concentrations were 0.5 μM for the *mip* primer set and 0.75 μM for the *dot* primer set. The optimization of the $MgCl_2$ concentration in the master mix indicated that between 3 and 4 mM was an optimal concentration for both primer sets. The Quantitect SYBR Green PCR kit (Qiagen, Hilden, Germany) provided a 4 mM $MgCl_2$ concentration.

The specificity of the SYBR Green assay was tested on a panel of *Legionella* and non-*Legionella* bacteria. The results showed positive signals (mean Ct values of 18.22) for all *Legionella pneumophila* bacteria (the reference strain and the environmental isolates). The amplification of the other *Legionella* species and non-*Legionella* bacteria produced only faint signals (Ct values higher than 35) and showed negative signals in the melting curve and agarose gel analyses.

3.3.2. Standard curve

A linear regression analysis was performed by plotting the Ct values against the logarithm of the copy number of each gene target. The experimental points aligned in a straight line with correlation coefficients (R) of -0.994 (R^2=0.9885) and -0.996 (R^2=0.9923) for *mip* and *dot*, respectively.

For *mip* gene detection, the equation from the regression curve was: Ct = -3.4135 log [GU] + 39.356. The slope of -3.4135 corresponded to an amplification efficiency of 96.32%. The assay showed a sensitivity of 9 GU per reaction (mean Ct = 35.3; Figure 3.1. A). The quantification limit was estimated to be 9.60 10^2 GU/L of sample. The coefficient of variation ranged from 1.7% to 2.8%, depending on the concentration of the DNA sample.

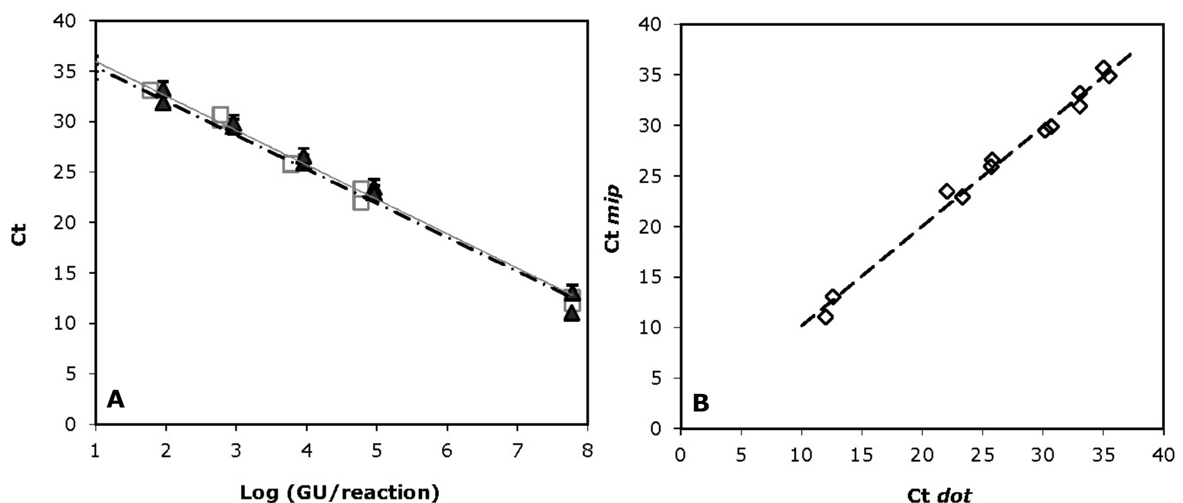


Figure 3.1. A. Standard curve for the mip (▲) and dot (□) genes. Serial 10-fold dilutions of *Legionella pneumophila* (Lp) DNA were amplified, and the standard curve was generated by a linear regression of the threshold cycles (Ct) versus the logarithm of *Legionella pneumophila* DNA concentration per qPCR reaction. GU: genome units. Ct: Threshold cycle. **B.** Correlation between quantitative SYBR Green based qPCR methods for detected Lp for *mip* and *dot* targets.

For *dot* gene detection, the equation from the regression curve was: Ct = -3.3698 log [GU] + 38.752. The slope of -3.3698 corresponded to an amplification efficiency of 98.04%. The assay showed a sensitivity of approximately 9 GU per reaction (mean Ct = 35.2; Figure 3.1.A). The quantification limit was estimated to be 9.58×10^2 GU/L of sample. The coefficient of variation ranged from 0.2% to 3.5%, depending on the concentration of the DNA template.

The *mip* and *dot* primers gave similar results for *Legionella pneumophila* quantification by qPCR. A comparison of the number of cycles required for detectable amplification with each primer showed a linear relationship from 6 to 6.00×10^6 GU/reaction (R^2 = 0.989; Figure 3.1.B).

3.3.3. Comparison between results from qPCR and *Legionella pneumophila* cultures

Pure suspensions of *Legionella pneumophila* were analysed by both qPCR and culture methods. The aim was to compare the quantitative results and determine whether they were correlated. A strong correlation was found between the positive, quantifiable results produced by the two real-time PCR methods (R^2 = 0.996; Figure 3.2.A).

Fifty water samples were analysed by culture and qPCR methods. Among these samples, 22 (44%) were culture positive and the remaining 28 (56%) were culture negative. The real-time PCR method indicated that, among the 22 culture-positive samples, 19 (86.4%) and 21 (95.4%) were positive for the *dot* and *mip* genes, respectively. Moreover, the real-time PCR showed that, among the 28 culture-negative samples, 7 (25%) and 10 (35.7%) were positive for *mip* and *dot*, respectively. The real-time PCR results were an average of 2.07 log higher than culture results for detecting the *mip* gene, with a margin of error of 0.3368 and a confidence interval (CI) of 95%. Similarly, the qPCR results were an average of 1.77 logarithms higher than culture results for detecting the dot gene, with a margin of error of 0.4306 and a CI of 95% (Figures 3.2.C and 3.2.B).

The melting curves and gel electrophoresis for both primer sets displayed single peaks and bands, respectively. In contrast, no peak or band was displayed in the negative controls. This indicated that these primer sets were specific for *Legionella pneumophila* (Figure 3.3).

To compare the culture qPCR methodologies, a linear regression was performed for all samples that had quantifiable amounts of *Legionella pneumophila* by both methods. A statistical analysis did not reveal any correlation between the two methods (R^2 = 0.137 for *mip* and R^2 = 0.234 for *dot*). However, there was a general association between a large number of colonies detected by culture and a high number of *Legionella pneumophila* genome units detected by real-time PCR.

Thirty hot tap-water samples were analysed. Among them, 14 (46.7%) were positive and 16 (53.3%) were negative by culture. Among the 14 culture-positive samples, 13 (92.8%) were positive for *mip* and *dot* by qPCR, and only one was negative by PCR. Real-time PCR results were, on average, 2.03 log higher than culture results for *mip* PCR, and 2.32 log higher than culture results for *dot* PCR. Among the 16 culture-negative samples, 5 (31.2%) and 6 (37.5%) were positive for *mip* and *dot*, respectively, by qPCR.

Twenty cooling tower samples were analysed. Among these, 8 (40%) were positive and 12 (60%) were negative by culture. Among the 8 culture-positive samples, 8 (100%) and 6 (75%) were positive for *mip* and *dot*, respectively, by qPCR. Real-time PCR results were, on average, 2.65 log higher than culture results for detecting the *mip* gene, and 2.10 log higher than culture results for detecting the *dot* gene. Among the 12 culture-negative samples, 2 (16.7%) were positive for *mip*, 3 (25%) were positive but non-quantifiable, and 1 (8.3%) was positive for *dot* by qPCR.

To compare the culture and qPCR methodologies, a linear regression was performed for all the samples that had quantifiable amounts of *Legionella pneumophila* by both methods. One regression was performed for the samples from each origin. The statistical analyses on both the sanitary and cooling tower water results did not reveal any strong correlations between

the culture and real-time PCR methods. But in general, the correlation for sanitary samples tended to be stronger than that observed for cooling tower samples.

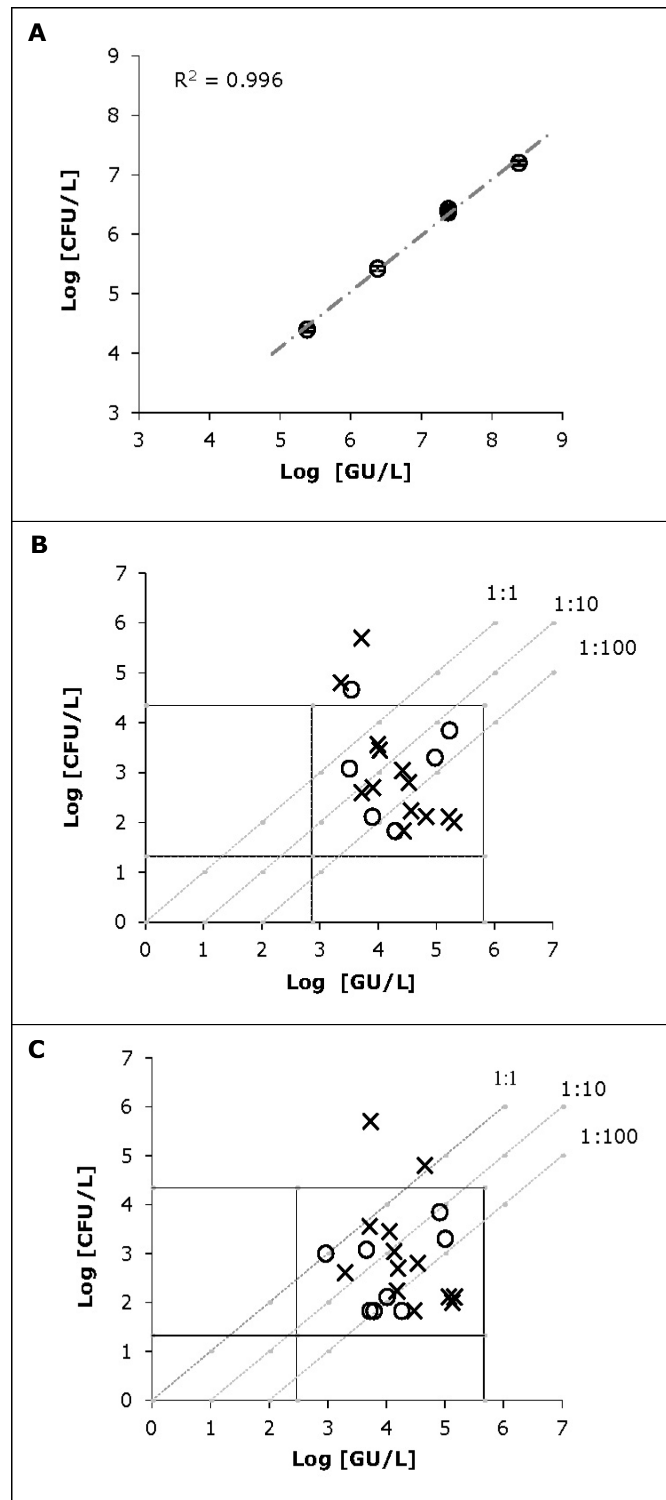


Figure 3.2. Correlation between the results obtained by SYBR Green based qPCR method and those obtained by the culture isolation technique for *Legionella pneumophila* (Lp). **A.** Dilutions of Lp pure cultures **B.** Water samples for primer *dot.* **C.** Water samples for primer *mip.* The symbol X corresponds to sanitary water samples and the symbol O corresponds to cooling water samples. GU: Genomic units.

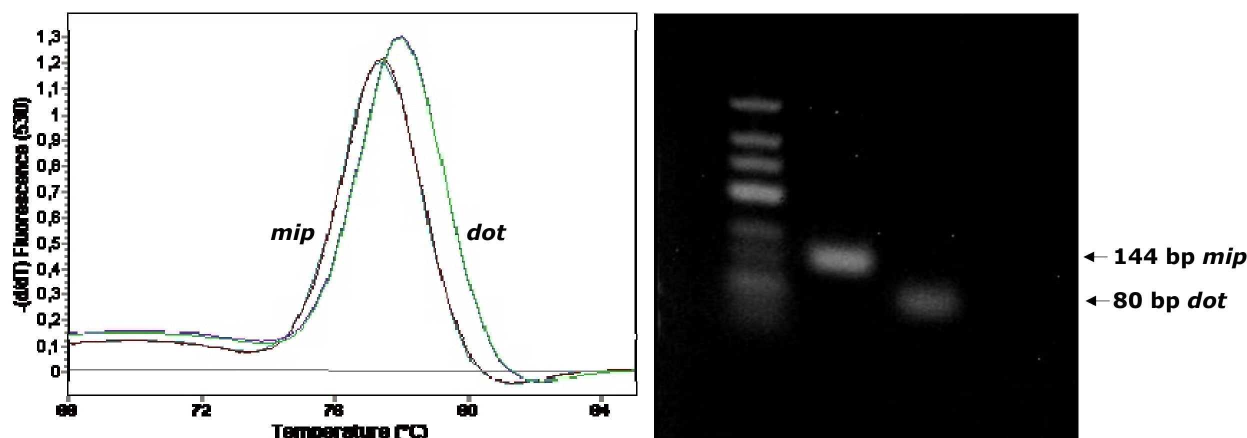


Figure 3.3. Melting curve and agarose gel electrophoresis for *Legionella pneumophila* using primers *mip* and *dot* as target. *Legionella* negative samples by qPCR did not show melting peak or it was very different from that of the positive control samples. In these cases any band was detected by electrophoresis.

3.3.4. Chi-Square Test (X^2)

Two-by-two contingency tables for each primer were constructed (Table 2). The X^2 was calculated for each table, considering one degree of freedom for both tables, and a *p*-value <0.01 (α = 0.01). According to the Chi-square distribution, for one degree of freedom and α= 0.01, the critical value is 6.63. Thus, when X^2 is greater than 6.69, the difference is significant, and the null hypothesis is rejected.

In this study, the X^2 were found to be 2.82 and 12.97 for *mip* and *dot* contingency tables, respectively. These results clearly showed that the culture and real-time PCR methods were correlated.

Table 3.2. Contingency table for *dot* and *mip* real-time PCR

Culture	qPCR *(dot)*		Total
	Positive	Negative	
Positive	19	3	22
Negative	10	18	28
Total	29	21	50
Culture	**qPCR *(mip)***		**Total**
	Positive	**Negative**	
Positive	21	1	22
Negative	7	21	28
Total	28	22	50

3.4. Discussion

A simple, sensitive, reliable qPCR method for detecting *Legionella pneumophila* in water samples is described in this chapter. We developed and optimized a real-time PCR assay based on SYBR Green and two pairs of primers that targeted the *mip* and *dot* genes. Three key features of PCR reactions were considered for optimization: the annealing temperature, the primer concentration, and the $MgCl_2$ concentration. Both primers were found to have the same high annealing temperature. A high annealing temperature is desirable because it enhances the specificity of the dye binding to the DNA avoiding primer-dimer formation. If the specificity is low there will be competition between the specific and non-specific qPCR product formation and it will reduce the assay sensitivity. A high specificity is always desired or required in a qPCR assay, but particularly when SYBR Green is used. As SYBR Green binds to any dsDNA, non-specific qPCR products will generate fluorescence leading to inaccurate quantification.

In order to quantify the number of copies of *Legionella pneumophila* in the water samples, a standard curve for each gene target was constructed. High amplification efficiencies were achieved for both the *mip* and *dot* primers (1.96 and 1.98, respectively). The amplification curves were highly correlated. Furthermore, a linear relationship was observed between the real-time PCR quantification of *Legionella pneumophila* by *mip* and *dot* primers; thus, both primers would be useful in a specific, fast, economical screening method for detecting *Legionella pneumophila* in water samples.

When pure cultures were analyzed, a strong linearity between the results achieved with SYBR Green qPCR with and those obtained with conventional culture techniques was observed. Joly et al. (2006) and Yañez et al. (2005) came to a similar conclusion. However, weak correlation coefficients were found between the results obtained by qPCR and conventional cultures when different environmental water samples (hot sanitary and cooling tower samples) were analyzed. Nevertheless, a tendency was observed towards higher correlation coefficients for hot sanitary samples than for cooling tower samples, particularly in detecting the *dot* gene. Several studies have reported similar results with real-time PCR (Wellinghausen et al., 2001; Yaradou et al., 2007; Joly et al., 2006; Morio et al., 2008).

Although no strong correlation was observed in the comparison of the two methods, the statistical analysis showed that a large number of colonies detected in culture was generally associated with a high number of genome units from *Legionella pneumophila* detected by real-time PCR. SYBR Green-based qPCR results were approximately, 1 logarithm and 2 logarithms higher than conventional culture results, for pure culture samples and both cooling tower and sanitary samples, respectively. Similar results have been observed in other studies (Wellinghausen et al., 2001; Yañez et al., 2005; Morio et al., 2008). Those differences may arise from various causes and can indicate diverse issues. The results from these methods are expressed in different units – CFU is the unit used in cultures while GU is used in qPCR. To the best of our knowledge, no methods have been established for deriving equivalent units for comparisons (StØlhaug and Bergh, 2006). Even though CFU values are not strictly comparable to GU values (Qin et al., 2012), usually, as was performed in this study, an equivalence could be reached by comparing real-time PCR and culture methods using pure *Legionella pneumophila* suspensions.

Wellinghausen et al. (2001) showed that DNA extraction enabled the detection of legionellae in free-living amoeba, but culturing methods could not detect amoeba. Furthermore, standard culture techniques based on ISO 11731 have numerous limitations (Behets et al., 2007; Qin et al., 2012; Yañez et al., 2011) and several factors complicate the interpretation of plate counting results (Devos et al., 2005). Cultures can be fastidious due to various factors, for example: the legionellae growth requirements necessitate prolonged incubation periods, the legionellae are difficult to isolate in samples contaminated with high levels of other microbiota particularly environmental samples, the pre-treatment by acid or heating can lead to underestimates of the number of viable legionellae, viable but non-culturable bacteria cannot be detected (Catalan et al., 1997; Wellinghausen et al., 2001; Yañez et al., 2005), and *Legionella* spp. are present a low densities in environmental samples. Therefore,

due to the mentioned culture technique limitations, the *Legionella* counts can be underestimated. Thus, the qPCR presents less risk of underestimating the number of cells present in the sample. On other hand, qPCR technique can detect all cells, both viable (culturable and non-culturable) and non-viable; by contrast, culture methods can only detect viable, culturable cells (Hussong et al., 1987). However, the inability to differentiate live from dead cells may lead to an overestimation of the infectious *Legionella*, and consequently, of the sanitary risk. It is worth of mention that some samples were positive by conventional culture while a negative amplification was observed by qPCR for the same samples. This could be explained for the presence of a low number of *Legionella* cells in those samples. Since the detection limit was higher for qPCR method (about 100 GU/L) than for culture (50 CFU/L). Moreover, the presence of PCR inhibitors, particularly in environmental samples, can result in false-negative results (Yañez et al., 2005; Yaradou et al., 2007). Regarding the primers, when *mip* gene was used less false-negative results were obtained (1) than those found using *dot* gene (3). However, few false-negative results were observed, and differences are not significant.

Although diagnostic methods have improved since *Legionella pneumophila* was first described in 1976, no test is currently available that can diagnose *Legionella pneumophila* in a timely fashion with a high degree of sensitivity and specificity (Diederen, 2008). Real-time PCR methods offer the benefit of speed over traditional culturing methods, and allow earlier disinfection of water systems that contain high numbers of *Legionella pneumophila* bacteria (Behets et al., 2007). The major disadvantage of qPCR lies in its inability to differentiate between viable and non-viable cells. This is important in monitoring *Legionella* contamination levels in environmental samples. So, there is an urgent need to add viability information to qPCR methods in order to implement them in routine applications in diverse fields ranging from testing of food and water safety to clinical microbiology (Fittipaldi et al., 2012). In the last years, several studies have reported the use of nucleic acid-binding dyes as an attractive alternative for selectively detecting and enumerating viable bacteria (Chang et al., 2009; Chen and Chang, 2010; Delgado-Viscogliosi et al., 2009; Qin et al., 2012; Yañez et al., 2011). This strategy to discriminate live from dead cells will be further studied and developed in the next chapters of this dissertation.

Occasionally, SYBR Green-based detection strategies in qPCR analyses have been criticized for their impracticality, due to the lack of dye specificity for binding to DNA (Hein et al., 2001). There is a common misconception that adding an oligoprobe to a reaction will automatically make the reaction more sensitive. An optimization step is usually needed, and specific and non-specific fluorogenic chemistries are able to detect amplicons with equal sensitivity (Newby et al., 2003; Fernandez et al., 2006). However, SYBR Green-based qPCR methods can require that the positive results be critically interpreted, especially in environmental samples were the target DNA/non-target DNA ratio will be low. The presence of primer-dimers or non-specific products can be detected in the melting curve analysis.

3.5. Conclusions

To the best of our knowledge, this was the first report on the use of real-time PCR in combination with SYBR Green for the quantification and identification of *Legionella pneumophila* in pure cultures and environmental samples. Good results in detecting *Legionella pneumophila* were achieved using an inexpensive, SYBR Green-based, quantitative, qPCR method. Furthermore, the results achieved were comparable to those obtained with real-time assays that employed expensive fluorescence resonance energy transfer probes (FRET). Non-specific chemical methods like SYBR Green are relatively inexpensive, do not require additional oligonucleotide design or chemical conjugation, and are minimally affected by small changes in template sequences. In contrast, small changes in template sequences can abolish the hybridization of an oligoprobe, even with primers that have previously amplified the template successfully (Komurian-Pradel et al., 2001).

Therefore, the optimized SYBR Green method appears to be a suitable alternative for monitoring *Legionella pneumophila* contamination, and should be economically feasible for large-scale routine testing. More studies that investigate real-time PCR assays for *Legionella pneumophila* detection are necessary to stimulate broader use of standard PCR methods. The detection and quantification of *Legionella* by qPCR could play key roles, both during an outbreak investigation and in the context of a health risk management program, especially if viability information is added.

3.6. References

AFNOR XP T90-471. 2006. Détection et quantification des *Legionella* et/ou *Legionella pneumophila* par concentration et amplification génique par reaction de polymérisation en chaîne (PCR). Normalization Française.

Anonymous. 1994. Legionnaires' disease associated with cooling towers – Massachusetts, Michigan and Rhode Island. Morb. Mortal Wkly Rep., 43, pp. 491–499.

Bartie, C., Venter, S.N., Nel, L.H. 2001. Evaluation of detection methods for *Legionella* species using seeded water samples. Water SA, 27, pp. 523-527.

Blatt, S.P., Parkinson, M.D., Pace, E., Hoffman, P., Dolan, D., Lauderdale, P., Zajac, R.A., Melcher, G.P. 1993. Nosocomial Legionnaires' disease: aspiration as a primary mode of disease acquisition. Am. J. Med., 95, pp. 16–22.

Behets, J., Declerck, P., Delaedt, Y., Creemers, B., Ollevier, F. 2007. Development and evaluation of a Taqman duplex real-time PCR quantification method for reliable enumeration of *Legionella pneumophila* in water samples. J. Microbiol. Methods, 68, pp. 137-144.

Casati, S., Gioria-Martinoni, A., Gaia, V. 2009. Commercial potting soils as an alternative infection source of *Legionella pneumophila* and other *Legionella* species in Switzerland. Clin. Microbiol. Infect., 15, pp. 571–575.

Catalan, V., Garcia Moreno, C., Vila, M.J., Apraiz, D. 1997. Detection of *Legionella pneumophila* in wastewater by nested polymerase chain reaction. Res. Microbiol., 148, pp. 71-78.

Chang, B., Sugiyama, K., Taguri, T., Amemura-Maekawa, J., Kura, F., Watanabe, H. 2009. Specific detection of viable *Legionella* cells by combined use of photoactivated ethidium monoazide and PCR/real-time PCR. Appl. Environ. Microbiol., 75, pp. 147-153.

Chen, N.T., Chang, C.W. 2010. Rapid quantification of viable legionellae in water and biofilm using ethidium monoazide coupled with real-time quantitative PCR. J. Appl. Microbiol., 109, pp. 623-634.

Chen, N.T., Chang, C.W., Wu, J.D. 2012. Evaluation of DNA isolation procedures for detecting and quantifying environmental *Legionella* by real-time quantitative PCR. Water Sci. Technol., 65, pp. 989-997.

Codony, F., Pérez, L.M., Adrados, B., Agustí, G., Fittipaldi, M., Morató, J. 2012. Amoeba-related health risk in drinking water systems: could monitoring of amoebae be a complementary approach to current quality control strategies?. Future Microbiol., 7, pp. 25-31.

Delgado-Viscogliosi, P., Solignac, L., Delattre, J.M. 2009. Viability PCR, a culture-independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. Appl. Environ. Microbiol., 75, pp. 3502-3512.

Devos, L., Clymans, K., Boon, N., Verstraete, W. 2005. Evaluation of nested PCR assays for the detection of *Legionella pneumophila* in a wide range of aquatic samples. J. Appl. Microbiol., 99, pp. 916-925.

Diederen, B.M.W. 2008. *Legionella* spp. and Legionnaires' disease. J. Infect., 56, pp. 1-12.

Diederen, B., Kluytmans, J.A.J.W., Vandenbroucke-Grauls, C.M., Peeters, M.F. 2008. Utility of real-time PCR for diagnosis of Legionnaires' disease in routine clinical practice. J. Clin. Microbiol., 46, pp. 671-677.

Fernández, F., Gutiérrez, J., Sorlózano, A., Romero, J.M., Soto, M.J., Ruiz-Cabello, F. 2006. Comparison of the SYBR Green and the hybridization probe format for real-time PCR detection of HHV-6. Microbiol. Res., 161, pp. 158-163.

Fittipaldi, M., Nocker, A., Codony, F. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J. Microbiol. Methods, 91, pp. 276-289.

Fleiss, J.L., Levin, B., Paik, M.C. 2003. Statistical methods for rates and proportions (3rd ed.). John Wiley & Sons, Hoboken, NJ, US.

Fliermans, C.B., Cherry, W.B, Orrison, L.H., Smith, S.J., Tison, D.L., Pope, D.H. 1981. Ecological distribution of *Legionella pneumophila*. Appl. Environ. Microbiol., 41, pp. 9–16.

Fliermans, C.B. 1996. Ecology of *Legionella*: from data to knowledge, a little wisdom. Microb. Ecol., 32, pp. 203–228.

Grabow, W.O.K. 1991. New trends in infections associated with swimming-pools. Water SA, 17, pp. 173-177.

Greub, G., Raoult, D. 2004. Microorganisms resistant to free-living amoebae. Clin. Microbiol. Rev., 17, pp. 413–433.

Hayden, R.T., Uhl, J.R., Qian, X., Hopkins, M.K., Aubry, M.C., Limper, A.H., Lloyd, R.V., Cockerill, F.R. 2001. Direct detection of *Legionella* species from bronchoalveolar lavage andopen lung biopsy specimens: Comparison of LightCycler PCR, in situ hybridization, direct fluorescence antigen detection, and culture. J. Clin. Microbiol., 39, pp. 2618–2626.

Hein, I., Lehner, A., Rieck, P., Klein, K., Brandl, E., Wagner, M. 2001. Comparison of different approaches to quantify *Staphylococcus aureus* cells by real time quantitative PCR and application of this technique for examination of cheese. Appl. Environ. Microbiol., 67, pp. 3122-3126. In: O'Mahony, J., Hill, C. 2002. A real-time PCR assay for the detection and quantitation of *Mycobacterium avium* subsp. *paratuberculosis* using SYBR Green and the LightCycler. J. Microbiol. Methods, 51, pp. 283-293.

Hussong, D., Colwell, R.R., O'Brien, M.O., Weiss, E., Pearson, A.D., Weiner, R.M., Burge, W.D. 1987. Viable *Legionella pneumophila* not detectable by culture on agar media. Bio/Technology, 5, pp. 947-950.

ISO, 2000. Water Quality – Guidance on Validation of Microbiological Methods. ISO/TR 13843:2000. Geneva: International Organisation for Standardization.

Joly, P., Falconnet, P.A., André, J., Weill, N., Reyrolle, M., Vandenesch, F., Maurin, M., Etienne, J., Jarraud, S. 2006. Quantitative real-time *Legionella* PCR for environmental water samples: data interpretation. Appl. Environ. Microbiol., 72, pp. 2801-2808.

Joseph C.A, Ricketts K.D. 2010. Surveillance and outbreak reports- Legionnaires' disease in Europe 2007–2008. EuroSurveill., 15, pp. 1-8.

Available: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19493

Kares, S., Lönnrot, M., Uvorinen, P., Oikarinen, S., Taurianen, S., Hyöty, H. 2004. Real time PCR for rapid diagnosis of entero-and rhinovirus infections using LightCycler. J. Clin. Virol., 29, pp. 99-104.

Komurian-Pradel, F., Paranhos-Baccalà, G., Sodoyer, M., Chevallier, P., Mandrand, B., Lotteau, V., Andrt, P. 2001. Quantitation of HCV RNA using real-time PCR and fluorimetry. J. Virol. Methods, 95, pp. 111-119.

Lin, H., Xu, B., Chen, Y., Wang, W. 2009. *Legionella* pollution in cooling tower water of air-conditioning systems in Shanghai, China. J. Appl. Microbiol., 106, pp. 606–612.

Mascone, C.F. 2008. Legionella: an invisible risk. Chem. Eng. Prog., 104, pp. 6-10.

Mastro, T.D., Fields, B.S., Breiman, R.F., Campbell, J., Plikaytis, B.D., Spika, J.S. 1991. Nosocomial Legionnaires' disease and use of medication nebulizers. J. Infect. Dis., 163, pp. 667–671.

Morio, F., Corvec, S., Caroff, N., Le Gallou, F., Drugeon, H., Reynaud, A. 2008. Real-time PCR assay for the detection and quantification of *Legionella pneumophila* in environmental water samples: utility for daily practice. Int. J. Hyg. Environ. Health, 211, pp. 403-411.

Murdoch, D.R., Podmore, R.G., Anderson, T.P., Barratt, K., Maze, M.J., French, K.E., Young, S.A., Chambers, S.T., Werno, A.M. 2013. Impact of routine systematic PCR testing on case finding for Legionnaires' disease: a pre-post comparison study. Clin Infect Dis. doi: 10.1093/cid/cit504.

Newby, D.T., Hadfield, T.L., Roberto, F.F. 2003. Real-time PCR detection of *Brucella abortus*: a comparative study of SYBR Green, I., 5'-Exonuclease, and hybridization probe assays. Appl. Environ. Microbiol., 69, pp. 4753-4759.

O'Mahony, J., Hill, C. 2002. A real time PCR assay for the detection and quantitation of *Mycobacterium avium* subsp. *paratuberculosis* using SYBR Green and the LightCycler. J. Microbiol. Methods, 51, pp. 283-293.

Qin, T., Tian, Z., Ren, H., Hu, G., Zhou, H., Lu, J., Luo, C., Liu, Z., Shao, Z. 2012. Application of EMA-qPCR as a complementary tool for the detection and monitoring of *Legionella* in different water systems. World J. Microbiol. Biotechnol., 28, pp. 1881-1890.

Rasmussen, R. 2001. Quantification on the LightCycler. In: Meuer, S., Witwe, C., Nakagawara, K. (Eds.) Rapid Cycler Real-Time PCR, Methods and Applications. Springer, Berlin. Germany, pp. 21-34.

Ratcliff, R.M., Lanser, J.A., Manning, P.A., Heuzenroeder, M.W. 1998. Sequence-based classification scheme for the genus *Legionella* targeting the mip gene. J. Clin. Microbiol., 36, pp. 1560-1567.

Rosmini, F., Castellani-Pastoris, M., Mazzotti, M.F., Forastiere, F., Gavazzoni, A., Greco, D., Ruckdeschel, G., Tartagni, E., Zampieri, A., Baine, W.B. 1984. Febrile illness in successive cohorts of tourists at a hotel on the Italian Adriatic coast: evidence for a persistent focus of *Legionella* infection. Am. J. Epidemiol., 119, pp. 124–134.

Somogyvari, F., Doczi, L., Serly, J., Ahmad, S., Nagy, E. 2007. Rapid discrimination between *Candida albicans* and *Candida dubliniensis* by using real-time polymerase chain reaction. Diagn. Microbiol. Infect. Dis., 58, pp. 367-369.

Steinert, M., Emody, L., Amann, R., Hacker, J. 1997. Resuscitation of viable but non-culturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. Appl. Environ. Microbiol., 63, pp. 2047–2053.

Stølhaug, A., Bergh, K. 2006. Identification and differentiation of *Legionella pneumophila* and *Legionella* spp. with real-time PCR targeting the 16S rRNA gene and species identification by mip sequencing. Appl. Environ. Microbiol., 72, pp. 6394-6398.

Wellinghausen, N., Frost, C., Marre, R. 2001. Detection of legionellae in hospital in water samples by quantitative real-time LightCycler PCR. Appl. Environ. Microbiol., 67, pp. 2047-2053.

Wójcik-Fatla, A., Stojek, N.M., Dutkiewicz, J. 2012. Efficacy of the detection of *Legionella* in hot and cold water samples by culture and PCR. I. Standardization of methods. Ann. Agric. Environ. Med., 19, pp. 289-293.

Yañez, M.A., Carrasco-Serrano, C., Barberá, V.M., Catalán, V. 2005. Quantitative detection of *Legionella pneumophila* in water samples by immunomagnetic purification and real-time PCR amplification of the dotA gene. Appl. Environ. Microbiol., 71, pp. 3433-3441.

Yañez, M.A., Nocker, A., Soria-Soria, E., Múrtula, R., Martinez, L., Catalán, V. 2011. Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. J. Microbiol. Methods, 85, pp. 124-130.

Yaradou, D.F., Hallier-Soulier, S., Moreau, S., Poty, F., Hillion, Y., Reyrolle, M., André, J., Festoc, G., Delabre, K., Vandenesch, J.E., Jarraud, S. 2007. Integrated real-time PCR for detection and monitoring of *Legionella pneumophila* in water systems. Appl. Environ. Microbiol., 73, pp. 1452-1456.

Yu, V.L. 1993. Could aspiration be the major mode of transmission for *Legionella*? Am. J. Med., 95, pp. 13–15.

Chapter 4

Evaluation of *Legionella* colonization in cooling tower demonstration systems when reclaimed water is used

Usefulness of qPCR technique as a tool for the monitoring of *Legionella pneumophila*

Promotion of reuse and water conservation is a key factor in a world which is attending to an important hydric resource crisis. Wastewater regeneration and reuse applications provide an opportunity for water conservation, and reduction of effluent discharges into receiving waters. Thus, reclaimed water contributes to guarantee the water availability in terms of both quantity and quality. Cooling systems are major water consumers in many industries; therefore, using reclaimed water for this purpose may bring considerable savings in fresh water consumption. However, operational and public health related problems are more challenging with the use of reclaimed water.

This chapter is based on research work performed to analyze *Legionella* colonization in cooling towers when reclaimed water is used. It was performed within the framework of a research project carried out together with the Agència Catalana de l'Aigua (ACA, Catalonian Water Agency), the Consell de Cambres de Comerç (Council of Chambers of Commerce) and a multidisciplinary research group of the Universitat Politècnica de Catalunya (UPC, Polytechnic University of Catalonia) and consisted of two stages. The first stage objective was to verify the effect of water origin in the colonization of cooling towers by *Legionella*. In order to achieve this objective, effluents of different sources from a wastewater treatment plant were used to feed a set of cooling tower demonstration units. The second stage objective was to evaluate the *in situ* disinfection process to diminish the sanitary risk of water reuse in cooling towers; and it was performed in a lab-scale cooling tower. Throughout the research project, both culture standard method and real-time polymerase chain reaction (qPCR) were implemented to detect *Legionella* in water and biofilm samples, with the aim of investigating the use of culture-independent alternatives to enable monitoring of microorganisms in water and wastewater reuse.

4.1. Introduction

In the last years, and especially in regions with scarcity of freshwater such as the Mediterranean countries, great attention is being paid into reclamation and reuse of wastewater from municipalities. To increase the use of non-conventional water resources a new environmental policy in Catalonia and Spain that promotes a new water culture is enforced. This plan seeks to increase the use of reclaimed water while the physical, chemical and microbiological quality of water is guaranteed. Thus the overexploitation of groundwater resources is avoided, and the recovery of the natural characteristics of surface waters is allowed (Cazurra, 2008).

After agriculture, industry is the second largest water user. Approximately 20 to 22% of global water demand is related to industrial use (Metcalf & Eddy, 1991; Wada et al., 2011; World Bank, 2001). However, it is important to note that industrial water withdrawal constitutes the biggest water withdrawal in industrialized high–income countries (Kohli and Frenken, 2011; World Bank, 2001). Reclaimed water can be reused by the industry sector as long as the water quality is suitable and the quality requirements established in the regulations, such as the Royal Decree (RD) 1620/2007 in Spain, are accomplished. Water industrial uses vary greatly and water quality requirements tend to be industry-specific. To provide adequate water quality, supplementary treatment may be required beyond conventional secondary wastewater treatment.

The largest single industrial use of water is for cooling; so cooling systems are prime candidates for reclaimed water usage (Rebhun and Engel, 1988). Recirculating cooling systems, such as cooling towers and its makeup water, are major water consumers in many industries such as power generation plants, petroleum refineries, chemical plants, general manufacturing, etc. Therefore, water availability is a growing concern in the industry field, and consequently it is very likely that new, reliable and abundant water sources for cooling will be needed in a near future (Li et al., 2011). Reclaimed water use for this purpose is very promising. Moreover, it may bring considerable savings in fresh water consumption.

Water quality requirements for industrial cooling are not generally as high as for other purposes (Wijesinghe et al., 1996). Operational and technical problems encountered for cooling towers can be categorized into three major areas: scaling, corrosion, and biological growth (Li et al., 2011; Rebhun and Engel, 1988). Actually, both potable water and reclaimed water contain contaminants, which can cause these problems. However, the concentrations of these pollutants are generally higher in reclaimed water (Li et al., 2011; Wijesinghe et al., 1996).

Regarding the legal and regulatory issues, the RD 1620/2007 is used to regulate today the practice of reclaimed water use in Spain. This decree allows the use of reclaimed water in industrial cooling tower that are placed in non-urban areas (Royal Decree 1620 Annex I.A.3, 2007). Total suspended solids (TSS) and turbidity are limited to a maximum of 5 mg/L and 1 Nephelometric Turbidity Unit (NTU), respectively, on a daily monitoring. *Legionella* spp. and *Escherichia coli* are also restricted; its absence is required based on three times per week monitoring schedule. Nematode eggs content is also regulated, being required its absence per liter based on weekly monitoring. Moreover, an authorization from the health authority of the control facilities specific program covered by Royal Decree 865/2003 is needed (Royal Decree 1620, 2007). Therefore, the use of reclaimed water for industrial cooling tower would actually be impeded in Spain, attaining the last approved regulation.

There exist other guidelines regarding the use of reclaimed water as makeup water in cooling towers. The Federal Environmental Protection Agency (EPA) has recommended a maximum limit of 30 mg/L per TSS (weekly monitoring), and also that the presence of fecal coliform bacteria should not be higher than 200 CFU/100 mL based on daily monitoring. The pH value should be maintained between 6 and 9 according to EPA guidelines (Li et al., 2011).

On the other hand, the Cooling Tower Institute (CTI) uses the heterotrophic bacteria population as a biological growth indicator stating a control criteria of 10^4 CFU/mL and 10^5 CFU/cm^2 for planktonic and biofilm bacteria, respectively (CTI, 2008).

Typical cooling tower operation conditions (25-45 ºC, moist environment, continuous aeration, and pH values between 6 and 9) are a favorable habitat for biological growth, and even more if organic matter is present. The presence of planktonic cells and, specially, the biofilm formation, are quite common issues in cooling tower systems, but also, become a major source of its performance deterioration (Qureshi and Zubair, 2006). This biological growth causes corrosion, and exacerbates mineral scaling problems leading to a progressive reduction in performance and efficiency (Ludensky, 2005). Moreover, it has the potential to cause human infection because of the potential for emission of contaminated aerosols that may pose environmental and health risks (Alonso, 2004; Dondero et al., 1980). Previous studies have shown a number of Legionnaires' disease outbreaks originating from cooling towers (Ferré et al., 2009; Garcia-Fulgueiras et al., 2003; Hugosson et al., 2007; Pastoris et al., 1997; Sabria et al., 2006). Therefore, routine monitoring of *Legionella* has proven to be an important strategy in prevention (Türetgen and Cotuk 2007). From a health risk perspective both planktonic and biofilm cells play an important role in cooling towers. Planktonic cells are of importance because of the potential for aerosol emissions, while biofilm cells constitute a reservoir of microorganisms that can be in the water system for a long time. According to Rebhun and Grynberg (1985), the attached growth comprises more than 80% of total biomass and its average residence times can reach a few weeks. Moreover, when biofilms are present a continuous release of microbial colonies back into the recirculating water can happen (Chien et al., 2012). Biofilms are directly related to re-contamination problems (Johansen et al., 1997), promoting resistance to antimicrobial compounds (Green and Pirrie, 1993; Kool, 2002), reducing heat transfer in thermal systems (Wright et al., 1991) and causing corrosion (Momba and Binda, 2002; Wijesinghe et al., 1996).

Finally, culture-independent techniques produce results faster than culture techniques, and in a context of microbiological risk assessment and public health prevention program, the delay to obtain results is not compatible with a real time management on the proliferation of the potentially pathogenic bacteria (Touron-Bodilis et al., 2011).

Considering all the above mentioned issues and with the aim of contributing to the use of reclaimed water in industrial applications, a study on reclaimed water use in cooling tower has been carried out. The main objectives of this study were the assessment of the importance of water origin in the *Legionella* colonization of cooling towers, the evaluation of the *Legionella* health risk through the analysis of its presence in the cooling water systems when reclaimed water is used and the possibility to control health risk with *in situ* disinfection, and the assessment of the usefulness of qPCR technique as a microbial water quality monitoring tool.

This study was performed in two stages: (1) analysis of the differences in *Legionella* colonization between types of water and then, (2) study of cooling system colonization when reclaimed water and *in situ* disinfection is used. Tests were performed only on demonstration units and a lab-scale system to study the microbial growth inside the recirculating systems. For health safety reasons a closed water recirculating system was used avoiding aerosol generation. In this sense, it is clear that the aeration that this system underwent is not the same that a large industrial scale cooling tower will have. However, air was allowed to enter the system by means of a lid on the cover which allowed for the colonization of aerobic microbiota.

Effluents from different stages of the wastewater treatment plant (WWTP) in Blanes (Girona, Barcelona, Catalonia, Spain) were used. Blanes WWTP has a wastewater treatment line and a sludge treatment line. The water treatment line consists of several stages encompassing: preliminary treatment, physico-chemical treatment, primary settling, biological treatment, secondary settling, and tertiary treatment. The latter involves various operations such as a physico-chemical treatment, a laminar settling, filtration by using sand filters, and finally a combined UV/chlorine disinfection.

4.2. Materials and Methods

4.2.1. *Legionella* colonization in cooling water demonstration units using different water sources.

In a first stage of the study, the importance of water origin in the *Legionella* colonization of cooling towers was analyzed working with demonstration recirculating units and four types of water.

Reclaimed water

Effluents of filtration, ultraviolet (UV) disinfection, and combined disinfection (UV +Chlorination, UV/CL_2) from the Blanes WWTP tertiary treatment were used as different water sources. Additionally, well water was used as control, making a total of four different water types to be analyzed. During the experimental studies using cooling tower demonstration units, the microbial quality analysis of the different water sources was performed. Samples were collected in 1 L sterile bottles. When necessary, sodium thiosulfate was added (30 mg/L) to the samples to neutralize chlorine treatment. Total aerobic bacteria, total coliforms, *Escherichia coli*, and *Legionella* spp. determinations were performed by conventional culture technique. Also qPCR technique was used to detect *Legionella pneumophila*. In addition, pH and electric conductivity (EC) were measured.

Cooling tower demonstration units

The experimental study was performed using four cooling tower demonstration units designed according to the scheme showed in Figure 4.1. Each of them consisted of an evaporative recirculating system and a reservoir of the water effluent to use from the WWTP. A 200 L tank was used as reservoir of the effluent to analyze from the WWTP, which was recirculated constantly with 80% renewal rate, in order to prevent water contamination by stagnation.

Each demonstration unit tank was made of fiberglass, and its dimensions were 34 cm width, 48 cm height and 40 cm length, with a total volumetric capacity of 70 L which was used by about 72%. Two electric heaters (Newatt, Alicante, Spain, 100 watts of power each), and an Eheim 1000 submersible pump (EHEIM GmbH & Co. KG, Deizisau, Germany) were placed in each tank (Picture 4.1.B).

The tank cover, reinforced with an aluminum plate, supported the column’s packing. The tank cover had a small opening window with an air filter, filled with active carbon, to ensure fresh air and daylight entry, and to simulate the evaporation that occurs in refrigeration equipment, but avoiding simultaneously potential health hazards (Picture 4.1.C). It also had a hole through which makeup water entered to the system. A pump and a control system with a height level sensor were used to regulate the water level in the tank. This pump ensured the replenishment of water that was evaporated from the system (Picture 4.1. A and Picture 4.1.C).

The recirculation circuit comprised two sections of flexible hose with outside diameter of 20 mm, inner diameter of 16 mm and a total length of 155 cm. Sections were easily removable, since the lower section allowed emptying the tank (or performing a blowdown). Moreover, a three-way valve onto the upper section allowed taking water samples for microbial analysis.

The packed column was built using a 1 m height and 160 mm diameter PVC pipe. It has a screw cap to allow packing sampling (Picture 4.2.A). Plastic rings with 16 mm diameter, 14 mm long, and specific surface of 0.7 m^2 were used as packing material, together with ceramic-filter rings (Wave®, Messina, Italy) with specific surface of 0.9 m^2, reaching a total area of approximately 327 m^2 (Picture 4.2.C). The packing was placed in three baskets constructed of plastic mesh (with a diameter of 150 mm and 300 mm long) and it was used for the characterization of biofilm (Picture 4.2.B). The baskets were placed one above the

other, and were supported by a 125 mm diameter and 140 mm high PVC ring, which lies directly on the basis of the column. Four channels were made in this PVC ring to achieve a good evacuation of the column, preventing packing flooding, and thus the operational problems that may derive therefrom.

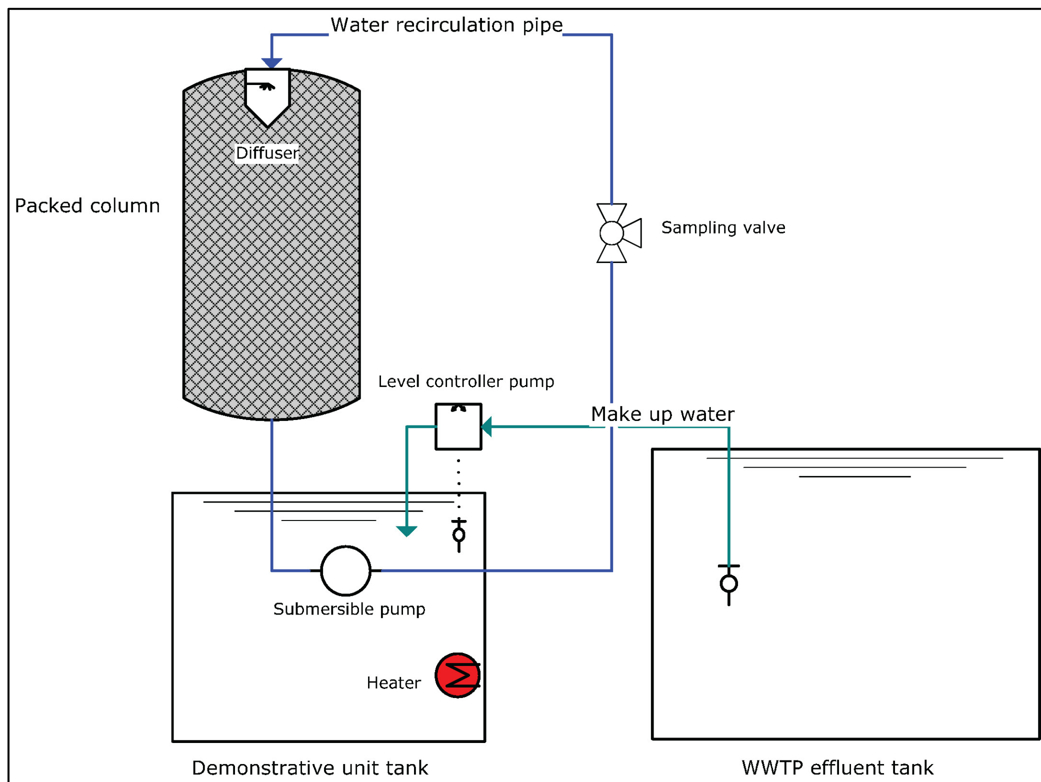
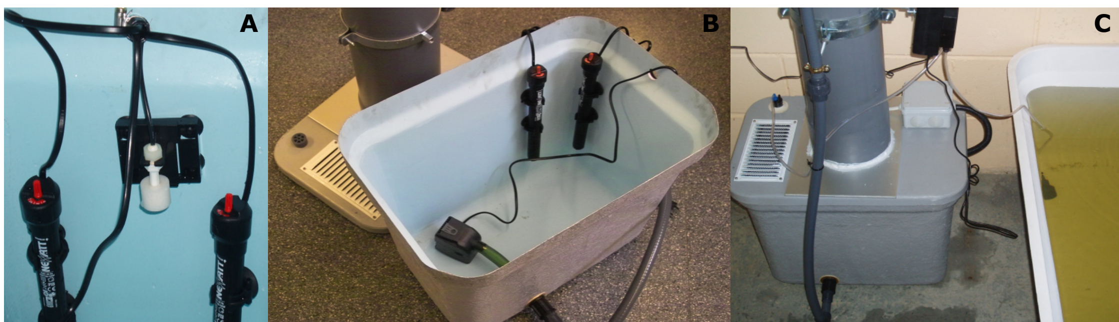
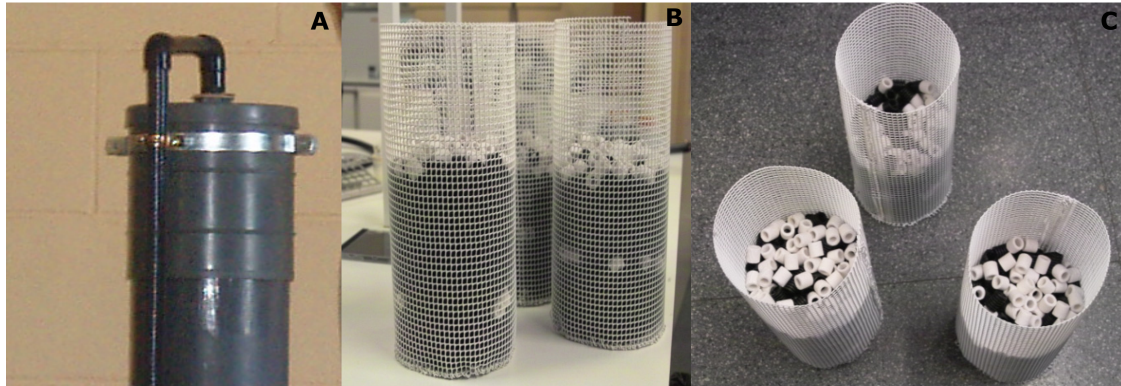


Figure 4.1. Design scheme of the cooling tower demonstration units. P: pump; V: valve.



Picture 4.1.A. Details of the demonstration unit tank: electric heaters and water level sensor. **B.** Deposit with the electric heaters and the submersible pump responsible for water recirculation in the system. **C.** Tank vent, pump to control the level in the system tank, and its connections.



Picture 4.2. A. Detail of the removable column's top connections and construction. **B.** Baskets used to contain the column's packing properly. **C.** Packing used in the column of each demonstration unit.

Demonstration units operation

In order to simulate the operation of a cooling tower, water from the different effluent tanks, filtration, UV disinfection, combined disinfection effluent, and well water- was heated to a temperature between 30 and 32 °C in the demonstration unit tank. Then water was recirculated by pumping it to the top of the column, where it passed through a diffuser (Picture 4.3). This diffuser allowed water falling as droplets through the packing and back into the tank. Demonstration units operated with 36 L/h as maximum working flow. This flow was controlled and set/regulated to achieve the greatest temperature difference (2-3 ºC). The blowdown (10% of the recirculating water) was performed monthly.

The demonstrative units were located at the Blanes WWTP (Picture 4.3), and the biological growth was monitored over a period of four months.

Picture 4.3. Cooling tower demonstrative units located to the Blanes WWTP. The large water reservoirs for each water type are located in the back.

Biological growth monitoring

Water and biofilm samples were taken fortnightly. Physico-chemical parameters -pH and EC- were determined *in situ* using a multiparameter probe (Multi 340i, WTW Inc., Weilheim Germany). Water samples were collected in sterile polypropylene bottles (1 L) and biofilm samples were collected in sterile polypropylene containers with 30 mL of sterile distilled water. Samples were transported to the laboratory at low temperature (8 ºC) within 5 h after sampling, and they were stored at 2-5 ºC. Samples were processed within 18 h after reception. With the aim to evaluate the microbiological water quality, microbiological analysis by conventional culture techniques were performed to detect total aerobic bacteria, total coliform bacteria, *Escherichia coli* and *Legionella* spp. Culture technique was also used to analyze the presence of aerobic bacteria in the biofilm. Real-time PCR was used to evaluate the presence of *Legionella pneumophila* both in water and biofilm samples.

Microbiological analysis in water samples

Culture technique

Total coliforms and *Escherichia coli* were determined by the method of membrane filtration according to the EPA. Water samples (100 and 10 mL) were concentrated by filtration using a nitrocellulose membrane filter (0.45 µm pore, Merck Millipore, Massachusetts, US). Filters were placed onto Chromocult agar plates (Merck, Madrid, Spain) supplemented with 10 mg/L Cefsulodin (Sigma Aldrich, Madrid, Spain). Plates were incubated at 37 ºC for 24 h.

The determination of total aerobic bacteria was performed according to ISO 6222 by plating 0.1 and 1 mL of different 10-fold dilutions of the sample on plate count agar (Merck, Madrid, Spain). Subsequently, plates were incubated at 22 ºC for 72 hrs.

Legionella spp. detection was performed according to ISO 11731:1998. Water samples (500 mL) were concentrated by centrifugation using a nylon membrane filter (0.45 µm pore, Merck Millipore, Darmstad, Germany). Filters were resuspended in 5 mL sterile saline solution (0.85% NaCl) into a sterile glass container, and some sterile glass beads (diameter, 5 mm) were added. Cells were detached from the membrane filter by vigorous vortexing for 60 s, followed by sonication for 3 min in an ultrasound water bath (40 W power, 40 kHz ultrasound frequency; JP Selecta, Barcelona, Spain). Acid buffer treatment was performed to reduce *Legionella* growth inhibition cause by commensal flora overgrowth. For that, 1 mL of the cell suspension was centrifuged (14500 rpm, 5 min) and 0.5 mL of the supernatant was discarded. The remaining 0.5 mL were mixed by vortex, and acidified with 0.5 mL buffered HCl-KCl solution (pH 2.2) for 5 min. From the acid treated sample, between 0.1 and 0.5 mL was spread on duplicate plates of *Legionella* selective medium (GVPC agar, Oxoid, Basingstoke, UK). These plates were incubated at 37 ºC and examined after 4, 7, and 14 days of incubation.

The buffered HCl-KCl solution (pH 2.2) was prepared by mixing 3.9 mL of a 0.2 M HCl solution with 25 mL of a 0.2 M KCl solution and adjusting the pH to a 2.2 value with a 1 M KOH solution.

Molecular technique

Legionella pneumophila detection was performed by qPCR technique. For that, water samples (500 mL) were concentrated by centrifugation using a nylon membrane filter (0.45 µm pore, Merck Millipore, Darmstadt, Germany). Filters were resuspended in 5 mL of sterile saline solution (0.85% NaCl) into a sterile glass container, and some sterile glass beads (diameter, 5 mm) were added. Cells were detached from the membrane filter by vigorous vortex for 60 s, followed by sonication for 3 min in an ultrasound water bath (40 W power, 40 kHz ultrasound frequency; JP Selecta, Barcelona, Spain). A pellet was obtained by centrifuging 4 mL of the cell suspension at 14500 rpm for 5 min using a MiniSpin centrifuge (Eppendorf, Hamburg, Germany). The supernatant was removed and the pellet was resuspended in 200

µL sterile PBS (1X, pH 7.4). DNA was extracted with DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Qiagen, 2006).

A qPCR protocol previously developed by our research group (Fittipaldi et al., 2010), described in Chapter 3 (section 3.2.5 *Quantitative PCR assays*), was used to detect *Legionella pneumophila mip* gene in water samples. However, for this study different commercial reagents, which allow faster analysis, were used (QuantiFast SYBRGreen, Qiagen, Hilden, Germany) whence slight changes were introduced in the experimental protocol. It was as follows: 2 min at 50 ºC to allow UDG to break down the possible contaminating amplicons, 5 min at 95 ºC for Taq-polymerase activation, followed by 45 DNA amplification cycles (95 ºC for 10 s of denaturing step, 60 ºC for 30 s of annealing and elongation step). Fluorescence data for melting curves were acquired by heating PCR products from 65 ºC to 95 ºC at 0.10 ºC/s.

The DNA used as standard reference for *Legionella pneumophila* quantification was prepared based on AFNOR XP T90- 471. *Legionella pneumophila* serogroup 1 (NCTC12821) was used as a reference strain. A standard DNA curve was established using a 3-day culture in GVPC agar (Oxoid, Basingstoke, UK). Once the culture was ready, a bacterial suspension was prepared by transferring single colonies into a tube with sterile saline solution and adjusting the optical density (OD_{600}) to 0.2, which approximately corresponds to a concentration of 10^8 cells per mL, as confirmed by plate count. Serial 10-fold dilutions were prepared from the bacterial suspension using sterile saline solution to obtain the set of dilutions that was later used for the standard curve. Pellets were obtained by centrifuging 1 mL of each dilution and DNA was extracted with DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Qiagen, 2006). Amplification efficiency was estimated by means of the slope calculation method from a calibration dilution curve (Rasmussen, 2001).

For each assay, the threshold cycle (Ct) was determined to quantify each DNA product. Quantification was performed including 1 or 2 external standards in each set of PCR experiments. The cell number of each sample was determined by comparison to each standard. Each sample was tested in duplicate and mean values were calculated. A negative control (PCR-grade water, QIAGEN, Hilden, Germany) was included in all assays.

A sample was considered negative when non-amplification was observed, or when there was amplification but the melting temperature was out of the range of the temperatures observed in previous amplifications, using the reference strain (79.4 ºC – 81.6 ºC). A sample was considered positive when the amplification of at least one of the replicas was produced and the melting temperature was inside the mentioned range.

Microbiological analysis in biofilm samples

Biofilm samples were rinsed twice in 50 mL sterile distilled water to remove unattached cells. Next they were suspended in 30 mL sterile saline solution (0.85%). Sessile cells were detached from the sampled rings by vigorous vortex for 60 s, followed by sonication for 5 min in an ultrasound water bath (40 W power, 40 kHz ultrasound frequency; JP Selecta, Barcelona, Spain).

For total aerobic plate count, 10-fold diluted biofilm homogenates were spread-plated (0.1 and 1 mL) onto plate count agar (Merck, Darmstadt, Germany). Subsequently, plates were incubated at 22 ºC for 72 hrs.

For *Legionella pneumophila* detection by qPCR technique, 4 mL of the biofilm homogenate were concentrated by centrifugation (14500 rpm for 5 min). The supernatant was removed and the pellet was resuspended in 200 µL sterile phosphate buffer saline (PBS, pH 7.4). DNA was extracted with DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (Qiagen, 2006), and samples were analyzed by following the qPCR experimental protocol descripted above in the *Microbiological analysis in water samples* – Molecular technique section.

Statistical analysis

Mean values and standard errors were calculated using Microsoft Excel. The results were statistically analyzed using the software Statgraphics 5.1 to test differences between water effluents. Significant differences are reported at the p-value <0.05 level, using the Student-t test.

4.2.2. *Legionella* colonization of a lab-scale cooling tower using *in situ* disinfection.

In a second stage of the study, a lab-scale pilot cooling system was used to assess the *Legionella* colonization when reclaimed water and *in situ* disinfection are utilized.

Lab-scale cooling tower

For this experiment, a lab-scale cooling water designed, constructed, and operated together by CRESCA and MSMLab research groups was used. The design was similar to that of the demonstration unit, but in this case, as the system was larger, a fan was used to draw air from the bottom of the tower to the top. The system was equipped with a 160 L tank, a recirculating pump and an immersion electric heater controlled by a thermostat. A PVC packing column with a diameter of 250 mm was used to simulate the tower. The packing material, with 255 m^2/m^3 specific surface, was constituted by Novalox saddles (VFF, Ransbach-Baumbach, Germany). Moreover, a drop separator and some filters were used to minimize the aerosol formation. Two level sensors were used to ensure proper operation of the heater and the recirculation pump.

Throughout the experiment, the work flow was between 150 and 160 L/h, the air velocity was 1 m/s, and the inlet water temperature was between 30 and 35 ºC. The cooling water temperature difference was approximately 2 ºC.

Reclaimed water

The UV disinfection effluent from the Blanes WWTP tertiary treatment was used in all the experiments. The water was collected in the WWTP and taken to the laboratory. The effluent was directly used or stored in a closed tank with agitation. The same water was used as makeup water, and no blowdown was performed during the experiments.

Experimental assays

Two *in situ* disinfection processes were evaluated during the experimental analysis, chlorination and a combined disinfection using UV and hydrogen peroxide (H_2O_2). The cooling system was cleaned and disinfected previously to each treatment. Also, both filters and packing were changed before each trial began.

Chlorination

The lab-scale cooling tower was operated for two months (end of February to end of April 2008). Chlorine (Cl_2) was applied continuously to reach a final free chlorine concentration of 1.5 mg/L during the three first weeks. Because no significant changes were observed in *Legionella pneumophila* colonization, the concentration was increased to 5 mg/L (Kim et al., 2002). Chlorine tablets with 1.5 gr of free Cl_2 per tablet (Millipore, Merck, Darmstadt, Germany) were used. Water and biofilm samples were taken weekly before disinfection treatment.

UV/H_2O_2 disinfection

The lab-scale cooling tower was operated for one month (end of July to end of August 2008). In this case, *in situ* disinfection was performed in the system tank where UV/H_2O_2 treatment was performed, and in the storage tank where only UV disinfection was carried out. Both tanks had an agitation system and were covered with foil to prevent loss of UV radiation.

Two UV systems (PURITEC LPS 9, OSRAM, Madrid, Spain) were used. The UV-C light intensity was 0.10 and 0.16 mW/cm^2 for the system and the storage tanks, respectively. Water irradiation was performed for 2 h every day. Regarding the peroxide, a concentration of 150 mg/L was used in four dosages of 10 mL every 5-10 minutes. The samples were collected before disinfection treatment with a frequency of 2 or 3 times per week.

Water sample analysis

Physico-chemical variables such as pH, EC and turbidity were monitored.

Microbiological analysis was conducted to investigate the occurrence of *Legionella pneumophila* in the cooling system. Samples were collected in 1 L sterile bottles. Sodium thiosulfate was added (30 mg/L) to the samples to neutralize chlorine treatment, according to the Standard Methods (1999). Determinations of total aerobic bacteria, total coliforms and *Escherichia coli* were performed by conventional culture technique following experimental protocol described above (section 4.2.1, *Microbiological analysis in water samples – Culture technique*).

Enterococci were also determined by membrane filtration methodology. Water samples (100 and 10 mL) were concentrated by filtration using a nitrocellulose membrane filter (0.45 µm pore, Merck Millipore, Darmstadt, Germany). Filters were placed onto Slanetz-Bartley agar (Merck, Darmstadt, Germany) and incubated at 37 ºC for 48 h. Colony confirmation of aesculin hydrolysis was performed by placing the filter into Bile Esculin Agar (Merck, Darmstadt, Germany) for 2 h at 44°C.

Legionella spp. detection in the chlorine *in situ* disinfection experiment was performed using the protocol descripted in the section 4.2.1 (Microbiological analysis in water samples – Culture technique). For *Legionella* spp. detection in the UV/H_2O_2 *in situ* disinfection experiment, water samples (500 mL – 1000 mL) were concentrated by centrifugation using nylon membrane filter (0.45 µm pore, Merck Millipore, Darmstadt, Germany). Filters were resuspended in 5 mL PBS into a sterile glass container, and some sterile glass beads (diameter, 5 mm) were added. Cells were detached from the membrane filter by vigorous vortexing for 60 s, followed by sonication for 3 min in an ultrasound water bath (40 W power, 40 kHz ultrasound frequency; JP Selecta, Barcelona, Spain). Acid buffer treatment was performed to reduce *Legionella* growth inhibition, produced by commensal microbiota overgrowth. For that, 1 mL of the cell suspension was acidified with 1 mL buffered HCl-KCl solution (pH 2.2) for 5 min. From the acid treated sample, between 0.1 and 0.5 mL was spread on duplicate plates of GVPC agar (Oxoid, Basingstoke, UK). These plates were incubated at 37 ºC and examined after 4, 7, and 14 days of incubation.

Real-time PCR technique was used to detect *Legionella pneumophila* applying the protocol detailed in section 4.2.1 (*Microbiological analysis in water samples* – Molecular technique). But in this case, the filters were resuspended in PBS instead of saline solution. Moreover, a pre-treatment of the sample using propidium monoazide (PMA), previously tested and based on Nocker et al. (2006) protocol, was performed to amplify mainly live cells.

PMA (Biotium, Inc., Hayward, CA, USA) was dissolved in a 20% dimethylsulfoxide (Sigma Aldrich, Madrid, Spain) solution, to obtain a 2 mM PMA stock solution. Concentrated pellet was resuspended in 500 µL of PBS in a propylene Eppendorf 1.5-mL tube and 12.5 µL of PMA stock solution was rapidly added in a darkened room. The resultant cell suspension was incubated for 5 min in the dark, at room temperature to allow PMA to enter into the cells with compromised or damaged membranes. The samples were then placed in ice bath to avoid overheating and were exposed for 2 min to a 650-W halogen light source (Quartzline Halogen Lamp, General Electric Company, Ohio, US) placed 30 cm from the sample tubes. After photo-induction of cross-linking, cells were pelleted in a minicentrifuge (minispin plus, Eppendorf, Hamburg, Germany) at 14500 rpm for 5 min, the supernatant was discarded, and the pellet was resuspended in 200 µL of 1X PBS to posterior DNA extraction and purification.

Biofilm samples were collected in plastic tubes containing sterile distilled water. Two 50 mL washes were performed using sterile distilled water. Finally, samples were placed in a second

plastic tube containing 15 mL of sterile distilled water added with 12.5 μL 3% solution of sodium thiosulfate as a neutralizing agent. In the UV/H_2O_2 assay 50 mL of sterile PBS were usedto resuspended attached cells. Sessile cells were detached from the sampled rings by vigorous vortexing for 60 s, followed by sonication for 5 min in an ultrasound water bath (40 W power, 40 kHz ultrasound frequency; JP Selecta, Barcelona, Spain). Total aerobic bacteria and *Legionella* determinations were performed according the protocols previously described (section 4.2.1 *Microbiological analysis in biofilm samples*). PMA pre-treatment was also applied in this case.

4.3. Results

4.3.1. *Legionella* colonization in cooling water demonstration units using different water sources.

<u>*Standard curve for qPCR assays*</u>

A linear regression analysis was performed by plotting the Ct values against the logarithm of the *Legionella pneumophila* cell number per reaction (see Figure 4.2). The amplification efficiency was 94%. The estimated quantification limit of this technique taking into account the volume of filtered water and the standard curve was $1.35x10^3$ CFU/L for water and $4.06x10^2$ CFU/cm^2 for biofilm. The detection limit was calculated to be $2.50x10^2$ CFU/L for water and 45 CFU/cm^2 for biofilm. This standard curve was used to quantify *Legionella* cells in subsequent studies. Based on these limits, samples were considered positive but non-quantifiable when the amplification was produced with a melting temperature within the considered range (79.4 ºC – 81.6 ºC), but with a Ct higher than 33.

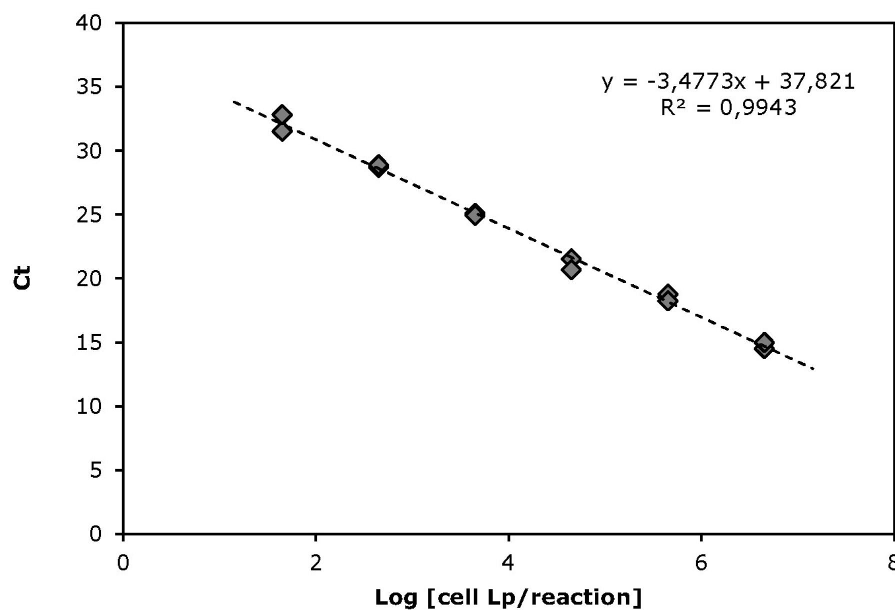


Figure 4.2. Standard curve for *Legionella pneumophila* (Lp) cells (CFU/mL) detected by qPCR.

Effluents water quality

The physico-chemical and microbiological variables measured during the experimental study for the four different effluents collected from the WWTP are depicted on Table 4.1. The quality of the four effluents was not significantly different in terms of pH and EC. However, the well water showed a lower EC than the other water sources. Regarding microbial quality, higher counts of *Escherichia coli* and total coliforms were observed in the effluent coming from filtration in comparison with those observed in the other effluents. The total aerobic counts were in the order of 10^4-10^5 CFU/mL. No significant differences were found between the different effluents, although higher values were obtained for well water samples and filtration effluent samples.

Table 4.1. Physico-chemical and microbiological characteristics of different water effluents. Each data represents the mean of 9 values ± the standard deviation measured on water samples collected between June and October of 2007.

Water Effluents	Well	UV/Cl_2	UV	Filtration
pH	7.4 ±0.2	7.2± 0.2	7.7±0.2	7.7±0.2
EC (µS/cm)	1,209.5±51.5	1,453±51.5	1,612.8±138.2	1,580.3±84.1
Total aerobic bacteria (CFU/mL)	$1.5x10^5$± $1.5x10^4$	$4.1x10^4$ ± $2.9x10^4$	$3.3x10^4$ ± $1.5x10^4$	$9.1x10^4$ ± $3.5x10^4$
Total coliforms (CFU/100 mL)	<10	<10	<100	99,000±31,000
Escherichia coli (CFU/100 mL)	<10	<10	<10	29,500±7,500
Legionella pneumophila (culture) (CFU/L)	Nd	Nd	Nd	Nd
Legionella pneumophila (qPCR) (CFU/L)	<$2.50x10^2$	<$1.35x10^3$	<$1.35x10^3$	<$1.35x10^3$

EC: Electric conductivity. Nd: results no determinable.

Although the presence of *Legionella pneumophila* was not detected in any case by conventional culture technique (detection limit of 50 CFU/L), positive but not quantifiable results were found in reclaimed water effluents using qPCR. *Legionella* was not detected in the well waters analyzed (detection limit $2.5x10^2$ CFU/L). It is worth to mention that this molecular technique detects both viable and non-viable cells. It is also important to remark that results obtained by culture technique were almost always difficult to be determined due to the presence of high levels of accompanying microbiota.

Microbiological quality monitoring in demonstrative units

The physico-chemical variables measured during the experimental study for the outlet waters from the different demonstrative units are depicted on Figure 4.3. The results were statistically analyzed, but no significant differences between the average values for the different quality waters studied were found.

Like in any water recirculating system that it is not controlled in terms of pH and EC, the values of the variables evaluated were higher for the outlet water than those for the inlet water. In this case, pH increased about 1 unit; and the EC increased approximately 12% in all demonstration units, except in the demonstration unit fed with well water in which the increment was only a 4%.

Mean values of total aerobic counts were in the order of 10^5 CFU/mL and 10^6 CFU/cm^2 for suspension and attached cells respectively, in the four demonstration units. The higher values were found in the system working with the filtration effluent. However, the statistical analysis of the results does not yield significant differences ($p = 0.33$ for water samples and $p = 0.59$ for the biofilm) between the different demonstration units water (Figure 4.4). The microbial load in terms of total aerobic bacteria was higher in the outlet water than in the inlet water for all the units.

For *Escherichia coli* and total coliforms determination, the results are summarized in Table 4.2.

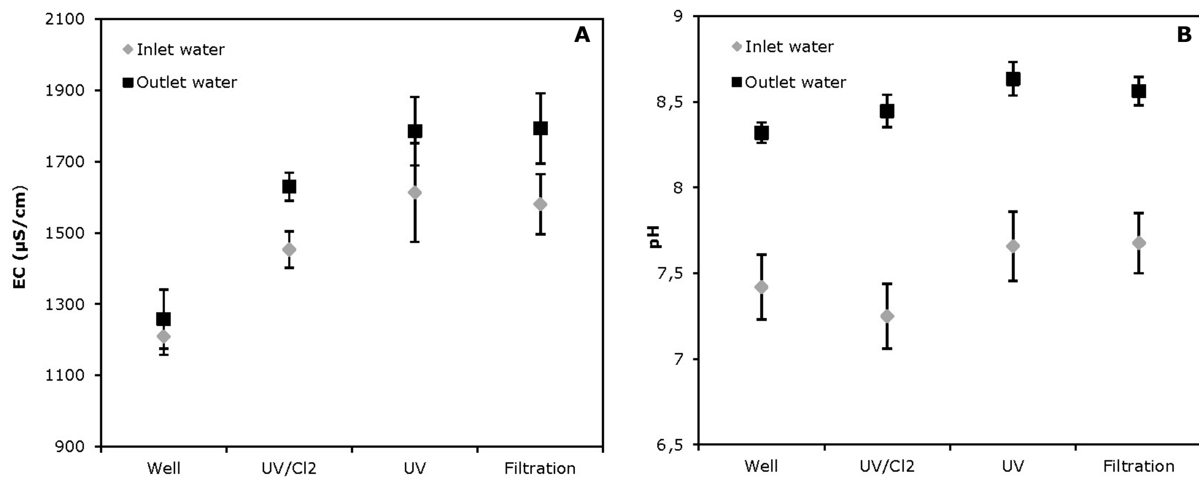


Figure 4.3. Physico-chemical quality of outlet water from demonstration unit. Each data represents the mean of 9 values ± the standard deviation. A: electrical conductivity (EC). B: pH.

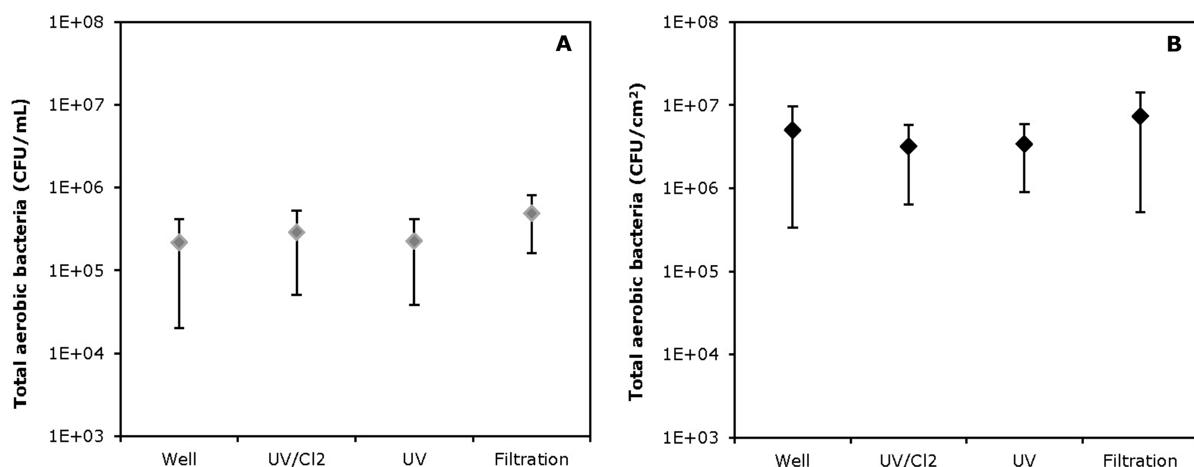


Figure 4.4. Microbial colonization of the different demonstration units, in terms of total aerobic bacteria load. A: in cell suspension analysis. B: in biofilm analysis.

Table 4.2. Microbiological quality, determined in terms of fecal bacteria, of the outlet water from the demonstration units. Each data represents the mean value of the plate counts ± the standard deviation.

Water Effluents	Well	UV/Cl_2	UV	Filtration
Total coliforms (CFU/100 mL)	<10	<10	<100	610±230
Escherichia coli (CFU/100 mL)	<10	<10	<10*	39±0.45

*In only one sample the count was 15 CFU/100 mL.

Regarding *Legionella*, some important problems in the detection and quantification by plate count analysis were identified. Most plates were unable to show quantitative results due to the quick overgrown of other bacteria (Picture 4.4). Despite this limiting factor, it was determined that all demonstration units were colonized by *Legionella pneumophila*. The approximate bacteria load was in the order of 10^3 CFU/L for the demonstration units fed with water from the well, or from tertiary disinfection steps (UV and UV/Cl_2), and 10^4 CFU/L for the demonstration unit recirculating water from tertiary filtration step.

The detection of this opportunistic pathogen was also performed by qPCR, obtaining similar results than with the culture technique. All demonstration units were colonized during the experimental study, and the demonstration units fed with well water or effluent from the disinfection steps (UV and UV/Cl_2) showed a similar average *Legionella pneumophila* load. It was approximately $3x10^3$ CFU/L for suspension cells and $1.6x10^3$ CFU/cm^2 for attached cells. On the other hand, the demonstration unit fed with the effluent from the filtration step showed an average load of $1.5x10^4$ cells/L for suspended cells and $1.4x10^3$ cells/cm^2 for biofilm (Figures 4.5 and 4.6). Even though no statistically significant differences were found in the analysis of the results obtained by qPCR for biofilm samples ($p=0.22$), statistically significant differences were found for the analysis of the results obtained for water samples ($p<0.05$). Effluent water samples from the demonstration unit fed with the effluent filtration and the demonstration units both fed with well water and effluents from the disinfection steps (UV and UV/Cl_2) showed statistically significant differences ($p=0.02$). Among the latter no significant differences were found.

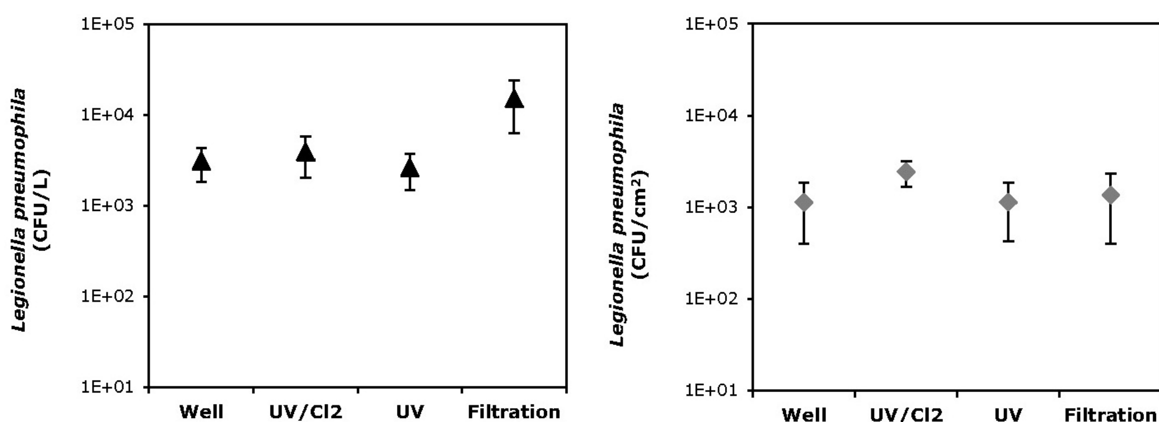
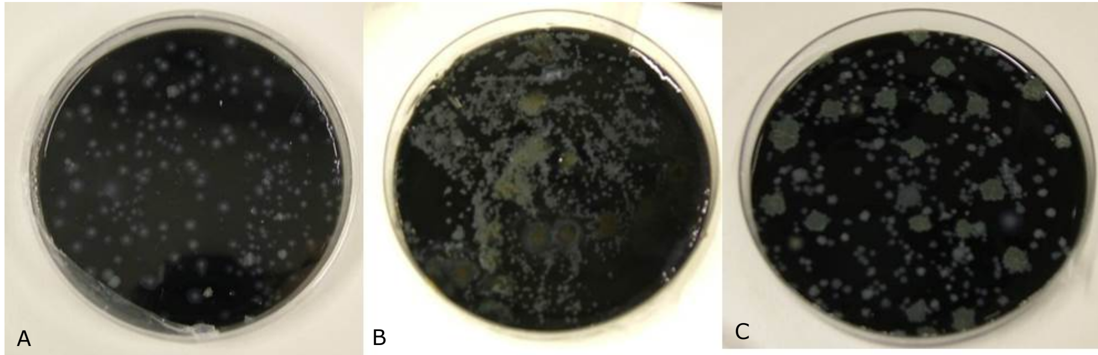


Figure 4.5. Mean *Legionella pneumophila* load values for the different demonstration units obtained by qPCR technique: A: water; B: biofilm. Each point represents the mean value of the quantifiable results ± standard deviation.



Picture 4.4. *Legionella* spp. plate counts. A: *Legionella* colonies can be perfectly identified and quantified. B: the results are undeterminable due to non-*Legionella* bacteria overgrowth. C: Some *Legionella* colonies can be identified but the results are non-quantifiable.

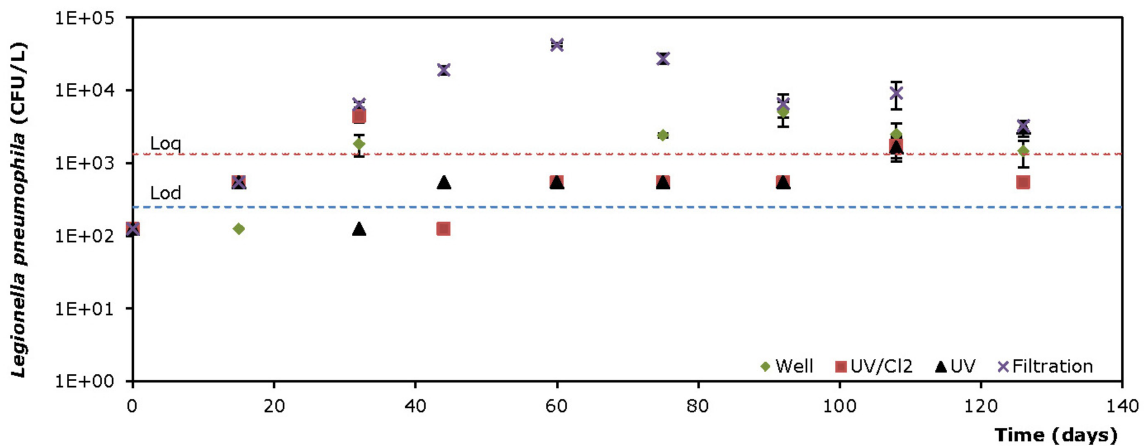


Figure 4.6. *Legionella pneumophila* colonization of the different demonstration units, analyzed by qPCR technique. Each point represents the mean of the cell number ± standard error. A representative value of 1.25×10^2 CFU/L was used when the obtained results was below the limit of detection (Lod = $2.50\ 10^2$ CFU/L). In the same way, a representative value of 5.5×10^2 CFU/L was used when the obtained results was below the limit of quantification (Loq = 1.35×10^3 CFU/L).

4.3.2. *Legionella* colonization of a lab-scale cooling tower using *in situ* disinfection.

In situ chlorine disinfection

The characteristics of used water are shown in Table 4.3. During the assay, *Escherichia coli*, enterococci and total coliform bacteria were not detected (<1 CFU/100 mL).

The counts for total aerobic bacteria were in the order of 10^4 to 10^5 CFU/mL or CFU/cm^2 for both planktonic and biofilm cells. Chlorination does not seem to affect the aerobic bacterial load in the system. However, when the chlorine concentration was changed from 1.5 mg/L to 5 mg/L, a one-log unit reduction was observed for suspended cells (Figure 4.7).

Regarding *Legionella* colonization, the system showed a high *Legionella* load when 1.5 mg/L of free chlorine was used. However, this load was progressively reduced when chlorine dose was increased. The achieved reduction was approximately 3 log unit (Figures 4.8 and 4.9). Similar results were observed for both, culture and PMA-qPCR techniques, although differences of 1 and 2-log units between the results obtained for both suspended and attached cells were observed for some analyzed samples.

Table 4.3. Physico-chemical and microbiological characteristics of the reclaimed water used in each experimental assay.

Variables	Reclaimed water Cl_2	Reclaimed water UV/H_2O_2
pH	8.35	8.07
Conductivity (µS/cm)	1,276	1,648.5
Total aerobic bacteria (CFU/mL)	1.4 10^5	100
Total coliforms (CFU/100 mL)	<1	<1
Escherichia coli(CFU/100 mL)	<1	<1
Enterococci(CFU/100 mL)	<1	<1
Legionella spp. (CFU/L)	3.1 10^4	<50
Legionella pneumophila (qPCR, CFU/L)	2.78 10^4	<250

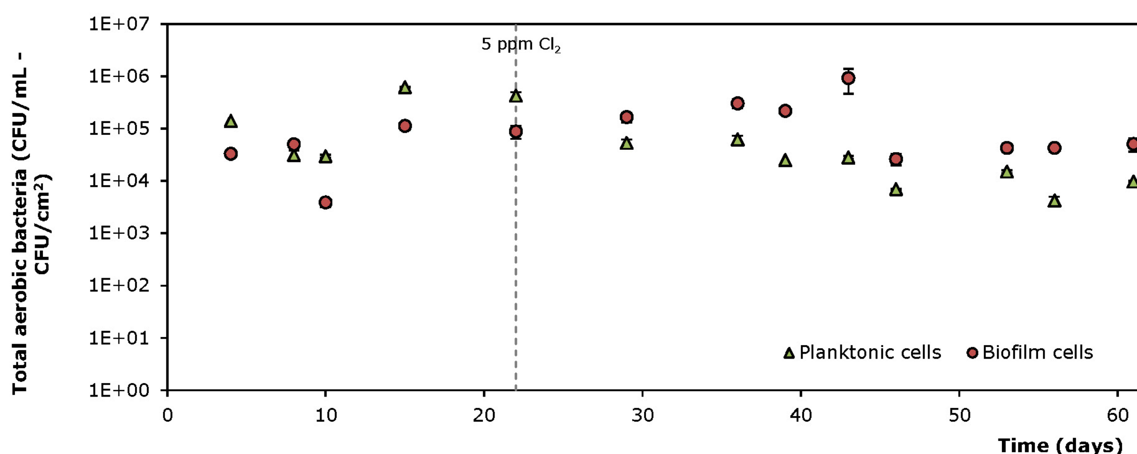


Figure 4.7. Total aerobic colonization (planktonic and biofilm cells) of the model cooling system, determined by culture technique. Each point represents the mean of duplicate values ± standard error. *In situ* disinfection using chlorine was applied. During the first 22 days a free chlorine concentration of 1.5 mg/L was used, and then it was increased to 5 mg/L. The detection limits were 5 CFU/mL and 2 CFU/cm^2 for planktonic and biofilm cells, respectively.

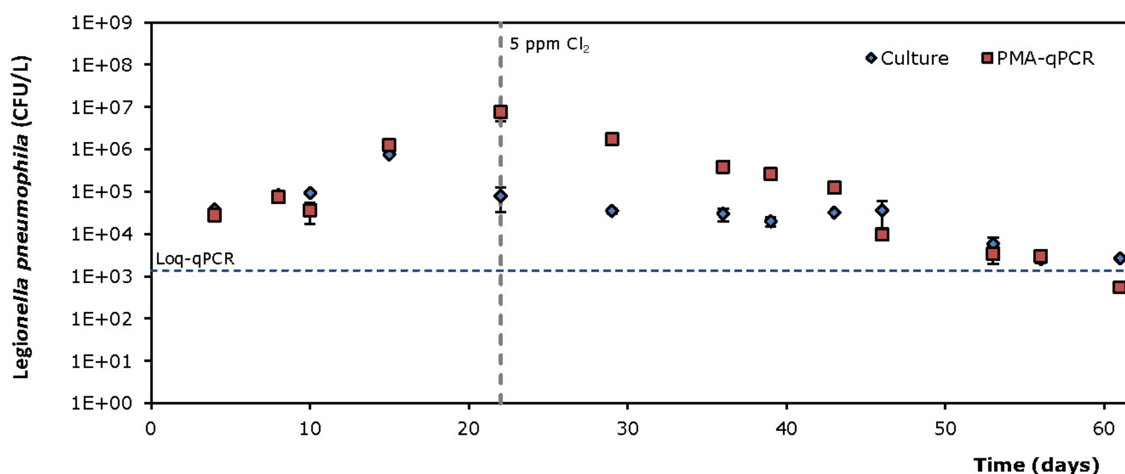


Figure 4.8. *Legionella pneumophila* colonization of the model cooling system, for water samples determined by both, culture and PMA-qPCR technique. Each point represents the mean of the Ct values or plate counts for duplicate samples ± standard error. A representative value of 5.5x10^2 CFU/L was used when the obtained results was below the qPCR limit of quantification (Loq-qPCR = 1.35x10^3 CFU/L).

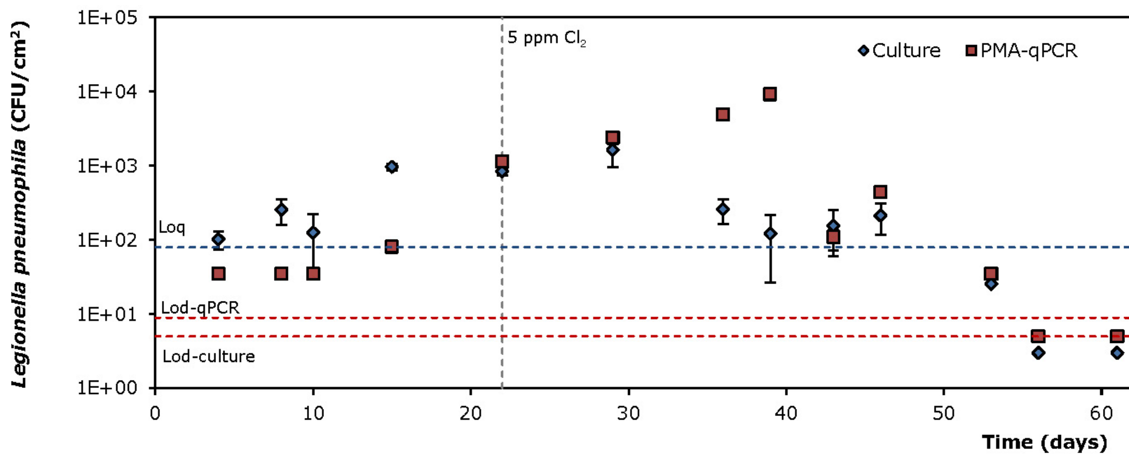


Figure 4.9. *Legionella pneumophila* colonization of the model cooling system for biofilm samples determined by both, culture and PMA-qPCR technique. Each point represents the mean of the Ct values or plate counts for duplicate samples ± standard error. A representative value of 36 CFU/cm^2 was used when the obtained results was below the qPCR limit of quantification (Loq = 80 CFU/ cm^2). The detection limit was 5 CFU/cm^2 for culture (Lod-culture) and 9 CFU/cm^2 for qPCR (Lod-qPCR); the adopted representative values were 3 and 5 CFU/cm^2, respectively.

The EC of the recirculating water in the system increased from 1,260 to 1,900 µS/cm during the experiment. Turbidity was highly variable, but values < 5 NTU were regularly observed, while pH values within the range of 7 to 9.

In situ UV/ H_2O_2 disinfection

The characteristics of used water are shown in Table 4.3. During the experimental assay the pH in both, the lab-scale cooling tower tank and the storage tank, was between 7 and 9. A decrease in pH was observed during the UV/H_2O_2 treatment. Conductivity in the storage tank increased from 1,299 µS/cm to 1,362 µS/cm, while it increased in the system tank from 1,315 to 2,110 µS/cm. Turbidity was between 1 and 2 NTU. Differences in the visual appearance of samples from both tanks were easily seen (Picture 4.5).

Picture 4.5. Appearance of the water samples from both, storage tank (on the right) and the system tank (on the left).

It is important to note that near the end of this study no disinfection was performed during four days, with the aim to study the residual effect of the *in situ* disinfection. In this case, samples were taken before and after the disinfection.

Escherichia coli and enterococci were not detected from water samples during the study (<1 CFU/100 mL) in both tanks. Regarding total coliform bacteria, no growth was detected in samples from the system tank. For the storage tank, presence of coliform bacteria was observed only after four days without in situ disinfection. The load before disinfection was 192 CFU/100 mL. However, after disinfection no coliform bacteria were detected (<1 CFU/100 mL). The following day before disinfection the load increased to 16 CFU/ 100 mL.

The aerobic bacterial load remained between 1 to 3.5 log units in the system tank, including the period of 4 days without disinfection. During this period, a load of 3.2 log units was observed before disinfection, and it decreased to 1.9 log units after disinfection. The microbial load reduction was 1.3 log units. The next days, an increase of 0.4 log unit was observed (Figure 4.10). The calculated mean value of load was 2.2 ± 0.3 log units. The minimum value was 1 log unit, and the maximum value was 3.5, and corresponds to a sample that did not receive any disinfection treatment the day before, due to operational problems.

Regarding the aerobic bacterial determination in the storage tank, the load was between 10^1 and 10^5 CFU/mL. Maximum values (5.3 log units) were reached when disinfection was not applied the day before to sampling. A reduction of 2 log units was observed when samples were taken before and after disinfection. An increment of 2 log unit was observed the next day before disinfection (Figure 4.10). In this case, the mean value of the microbial load was 3.2 ± 0.6 log units.

Biofilm formation was controlled in the system tank. The microbial load in terms of total aerobic bacteria was between 1 and 3.4 log unit, being the mean value of 1.5 ± 0.3 log units (Figure 4.11).

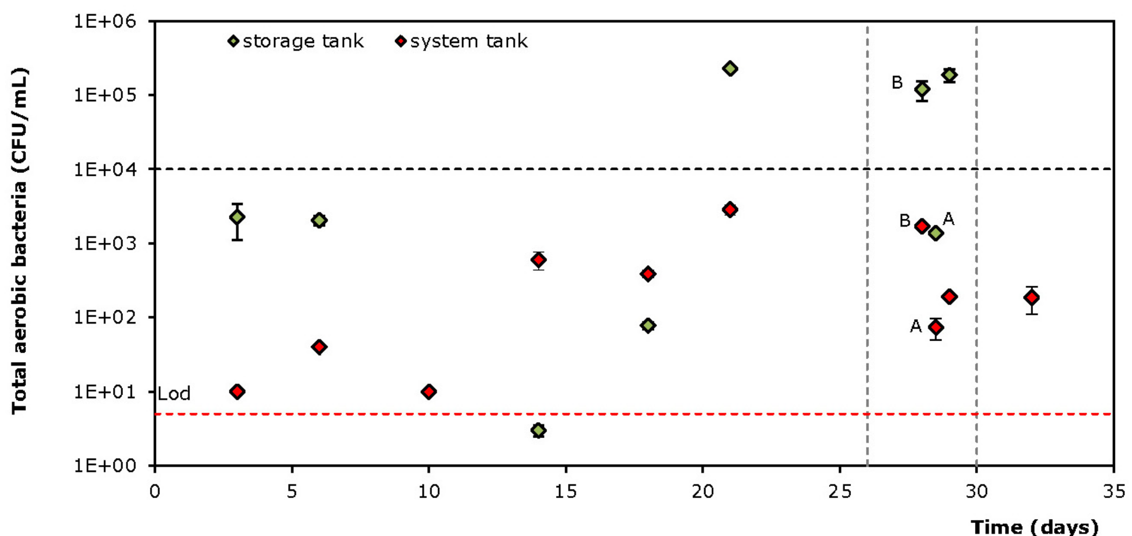


Figure 4.10. Total aerobic colonization of both, system and storage tanks, for water samples by culture technique. Each point represents the mean of duplicate values ± standard error. In situ disinfection using UV/H_2O_2 was applied. Lod: limit of detection, 5 CFU/mL. B: sampling before disinfection. A: sampling after disinfection. The black dashed line delimits the found bacterial concentration in the system tank throughout the study.

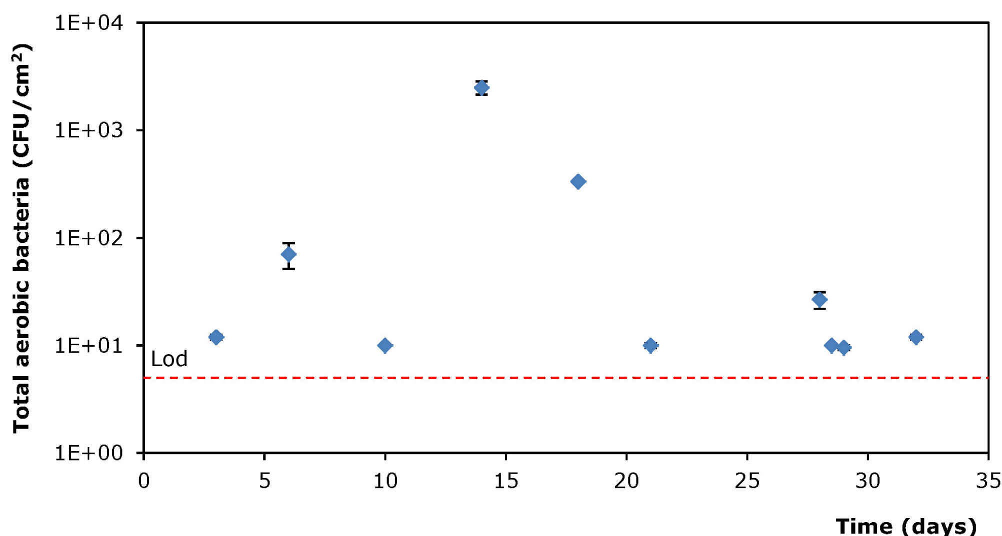


Figure 4.11. Colonization of the model cooling system in terms of aerobic biofilm bacteria by culture technique. Each point represents the mean of duplicate values ± standard error. In situ disinfection using UV/H_2O_2 was applied. Lod: limit of detection, 5 CFU/cm^2. B: sampling before disinfection. A: sampling after disinfection.

Planktonic or attached *Legionella* cells were not detected by culture, and neither by PMA-qPCR. Detection limits were 100 CFU/L and 12 CFU/cm^2 for planktonic and biofilm cells, respectively, by culture technique and 250 CFU/L and 30 CFU/cm^2 for planktonic and biofilm cells, respectively, by PMA-qPCR technique.

4.4. Discussion

The ultimate purpose of the first stage of this study was to analyze whether the use of reclaimed water in cooling towers is associated or not to higher health risk depending on the water sources used, and also to analyze the usefulness of the qPCR technique as a tool for the monitoring of *Legionella* in these systems. In order to reach these objectives four cooling water demonstration units were designed, constructed, and operated during four months. Effluents from three different steps of the tertiary treatment- sand filtration, UV, and UV/Cl_2 disinfection- from Blanes WWTP were used to feed each of the demonstration units. Moreover well water was used as control, since many companies use this kind of water in their cooling systems.

The operation of the systems proved to be challenging. Although problems associated with pumping failures and water leaks had to be resolved during the experimental assay, the integrity of the whole experiment data set was maintained.

The physico-chemical and microbial quality of the different used effluent was within of the range of values observed in previous studies for secondary and tertiary treated wastewater effluents (Cirelli et al., 2012; Pedrero and Alarcón, 2009; Varma et al., 2009). However, it is important to note that the quality of treated wastewater depends on the nature of the wastes added during its use and the quality of the municipal water supply (Pedrero et al., 2010). The pH values for all the monitored systems were between 6 and 9 as recommended by EPA, and the EC did not show a large increase during the experimental assays. The increase of EC is typical of concentration loops where water leaves the system, thus increasing the total number of charged species left in the system. The mean value for TSS and turbidity in the

Blanes WWTP effluent (UV/Cl_2) during the experimental period were 1.8 mg/L and 1.7 NTU, respectively. For the secondary effluent (influent to the filtration step) they were 4 mg/L for TSS and 2.4 NTU for turbidity (data supplied by the Blanes WWTP laboratory). For both, secondary and tertiary effluents, TSS values were always below 5 mg/L, so they meet the demanding value for regulation (RD1620/2007). Regarding the turbidity values, for both, secondary and tertiary effluent, they were in all cases higher than 1 NTU (set value in the RD1620/2007). However, turbidity values higher than 2.5 NTU were not observed during the experimental period. This brings to the table the need to discuss the demanding quality criteria imposed on the use of reclaimed water. In some cases, such as the turbidity requirements for cooling tower, these criteria are equivalent to those established for drinking water (Royal Decree (RD) 140/2003).

Regarding the microbial quality requirements for reclaimed water use in cooling tower, in this case, it was observed that they could be met only if a disinfection treatment is used.

Total aerobic counts were 1 log unit higher than those proposed as control criteria (10^4 CFU/mL and 10^5 CFU/cm^2) by the CTI (2008). Moreover, it is important to note that in this case no additional disinfection treatment was applied in the recirculating systems. Statistically significant differences between the total aerobic loads, both on suspension and attached cells, of the different units were not found. However, the system working with the filtration effluent provided higher levels of biological growth. This is reinforced by the results obtained in the counts of total coliforms and *Escherichia coli*, as it was the only demonstration unit that showed fecal contamination. It is important to note that for the unit fed with reclaimed water from the filtration step, the *Escherichia coli* and total coliform mean load was lower in the outlet water than in the inlet. A possible explanation could be the short survival of these microbiological indicators, compared to other microorganisms that have already been shown by other researchers using pond or well water as work matrix (Jenkins et al., 2009; McFeters et al., 1974). Lehtola et al. (2007) also demonstrated that *Escherichia coli* has a short survival and it is not a good indicator of certain pathogenic bacteria, such as *Legionella pneumophila*, both in water and biofilms. Ajibode et al. (2013) have shown that water-based opportunistic pathogens (*Legionella*, *Mycobacterium*, and *Aeromonas*) were frequently detected in reclaimed water systems, while in contrast, waterborne indicators such as *Escherichia coli* and *Enterococcus* were rarely detected, and only at low concentrations. This emphasizes the need to develop methods for detecting and enumerating the pathogens themselves (Jenkins et al., 2009).

The four demonstration units analyzed showed a positive colonization by *Legionella pneumophila* during the follow-up of the demonstration units, regardless the water source. However, significant differences were found between the *Legionella* load in water samples for the demonstration unit recirculating water from filtration, and the *Legionella* load in the other systems analyzed. It should be noted that among the demonstration units fed with well water and reclaimed water from UV disinfection and UV/Cl_2 disinfection, no statistically significant differences were found. These results highlight the importance of the disinfection treatment for wastewater reclamation. In fact, the results suggest that, under the conditions studied, reclaimed water treated with some kind of disinfection step behaves equivalent to untreated natural water with regard to the risk of *Legionella* colonization.

Regarding biofilm tests, no statistically significant differences were observed between *Legionella* colonization in the four demonstration units. This finding focuses the importance played by biofilms as the most important role in the microbial dynamics in this kind of installation. Biofilms constitute bacterial niches that are often hard to eradicate, so it is very important to devise disinfection processes to eliminate attached cells.

In the second stage of this study two experimental studies were conducted to analyze the microbial colonization of a lab-scale cooling system when reclaimed water, previously treated with UV, is used. *In situ* disinfection treatment was used for each study, chlorination (1.5 and 5 mg/L) and UV/H_2O_2.

Important differences in the microbial quality of the reclaimed water used in each experiment were noticed. The water used in the chlorine *in situ* disinfection assay showed a high microbial load in terms of total aerobic bacteria (10^5 CFU/mL) and *Legionella* (10^4

CFU/L). Thus, it did not meet the quality requirement established by regulations and guidelines. On the other hand, the water used for the UV/H_2O_2 test met, to a considerable extent, the quality recommendations of guidelines and regulation. Total aerobic load was lower than 10^4 CFU/mL as it is recommended by the CTI (2008), and *Escherichia coli* and *Legionella* were not detected accomplishing the RD1620/2007 requirements. These numbers showed the great quality variability, which can be found when reclaimed water is used as an alternative water source. The variability in the abundance of microorganisms in reclaimed water has also been observed by other researchers in previous works (Alonso et al., 2006; Cirelli et al., 2012, Palese et al., 2009). This variation could significantly impact to the water end-user and is one of the main concerns about reuse of treated wastewater (Higgins et al., 2002).

No differences were observed when chlorine was dosed at 1.5 mg/L to control microbial colonization, the contrary with a 5 mg/L chlorine dose. Total suspended aerobic bacteria load was slightly reduced when the used chlorine dose was 5 mg/L. Biofilm load showed higher variability and resistance to disinfection. It is important to note, that in this test no *in situ* disinfection was applied in the water storage tank, so it is highly probable that the used makeup water was contaminated, making difficult the microbial contamination control in the cooling system. The *Legionella* proliferation was not suppressed by 1.5 mg/L chlorination. Chlorine concentrations of 2 to 6 mg/L were needed to continuously control *Legionella pneumophila* in water distribution systems according to the results reported by Lin et al. (1998). Under the study conditions, a free chlorine concentration of 5 mg/L was necessary to control biological growth when highly contaminated reclaimed water was used in the lab-scale cooling system. Absence of *Legionella* was not achieved during the assay, although seems like it could be reached if 5 mg/L chlorine dose is used from the start of the test. In previous works, continuous dosing with chlorine to eliminate *Legionellae* from water systems did not show to be effective (Pankhurst et al., 1990). Carducci et al. (2010) have studied different *Legionella* control strategies in industrial cooling towers. They found that inactivation and suppression of the bacterium require residual chlorine levels of at least of 3 mg/L. Chlorine shocks resulted to be insufficient to control *Legionella* contamination in cooling waters and water distribution systems because it only reduced the concentration of bacteria temporarily due to rapid dissipation of chlorine, and subsequent regrowth (Ajibode et al., 2013; Carducci et al., 2010). It is worth of mention that a stable residual chlorine concentration is often difficult to maintain because of changes in incoming water quality, so this can be a reason to explain the high needed chlorine concentration. Furthermore, chlorine can decay faster at high temperature (Muraca et al., 1987).

The need of a high chlorine concentration to control *Legionella's* growth is a disadvantage because the materials of construction used in the chlorinated water systems can be affected by the corrosiveness of chlorine; as well as a high formation of harmful disinfection by-products. Taking into account the above mentioned, it is important to study other strategies to control *Legionella* in water recirculating systems.

In this study UV/H_2O_2 combined disinfection was used to control *Legionella* contamination in a lab-scale cooling tower recirculating reclaimed water. The results obtained in this experiment were very positive in terms of water reuse. This may be connected to the initial good water quality and also because UV disinfection was performed in the storage tank. The total aerobic bacterial load for both, planktonic and biofilm, were lower than the CTI (2008) recommended levels, 10^4 CFU/mL and 10^5 CFU/cm^2 respectively. Moreover, *Legionella* was not detected during all experiment (<100 CFU/L). Preliminary results indicated that, different than UV treatment, disinfection combining UV and H_2O_2 has a residual effect at least in the *Escherichia coli* and total aerobic bacteria load. The lack of disinfectant residual, the interference with UV light transmittance by turbidity (Kim et al., 2002), and the potential risk of repair of some microorganisms, such as *Legionella*, after UV disinfection (Oguma et al., 2004) greatly impair the efficacy of UV as microorganisms control treatment. Accordingly, a combination of UV with other disinfection method, such as H_2O_2 would be recommended for an effective control of *Legionella*.

It is noteworthy to mention the difficulties encountered in the detection and quantification of *Legionella pneumophila* using the conventional culture technique, especially during the first

stage of this study. Although acid treatment of the samples was performed before plating them, a large number of results were indeterminable. This is usual for environmental samples, such as natural water and reclaimed water, where the presence of high levels of other microbiota hampers *Legionella* isolation (Devos et al., 2005; Yañez et al., 2005). The issue that problems associated with *Legionella* determination by culture were less for the *in situ* disinfection study could be related with the use of on-site disinfection, which reduces the non-*Legionella* bacteria population. Even though culture is the standard technique for detection of *Legionella* in water, it has many limitations adding difficulties to both obtaining the results and interpreting them. Moreover, it is time-consuming and long incubation periods are required (at least 10 days) to get reliable results. The latter is a serious drawback when both daily operational and health risk associated decisions must be taken.

To support fast decision making, the use of molecular detection techniques such as qPCR acquires a truly remarkable importance. Several studies have shown that this technique allows detecting, in a short time (2 to 3 hours), the presence of *Legionella* spp. and *Legionella pneumophila* in water (Behet et al., 2007; Kao et al., 2013; Wellinghausen et al. 2001; Yañez et al. 2005). The speed, specificity, and sensitivity of this technique allows for taking preventive and corrective measures, such as disinfection, in an expeditious manner avoiding possible outbreaks.

Real-time PCR, as well as other microbial detection techniques, has some drawbacks. One of the most important is that, being a DNA amplification and detection based technique, both viable cells and non-viable are detected at the same time. This issue is of paramount importance given that it could thwart the health risk assessment. In the last years, the use of DNA intercalating substances such as ethidium and propidium monoazide (EMA and PMA), have been successfully used for the differentiation of viable and non-viable cells (Chang et al., 2008; Nocker et al., 2006; Rudi et al., 2005; Yañez et al., 2011). This progress made qPCR technique more valuable and increased its usefulness as microbial detection tool. In this study the new viable PCR technique was applied, following a previous reported protocol (Nocker et al., 2006). This technique showed to be usefulness to monitor the disinfection processes, as well as other authors have previously determined (Nocker et al., 2007; Wahman et al., 2009). However, although PMA-qPCR results agreed with plate count results to a quite extent, important differences were found for some samples. Further experiments to explain these differences will be required. However, it is important to take into account that PMA-qPCR theoretically detects viable cells both, culturable and non-culturable, while culture only detects viable culturable cells. Under harsh environmental conditions, such as most usual disinfection treatments, bacteria can enter in non-culturable state but still be alive. Moreover, further optimization of the PMA-qPCR or viable-qPCR technique is needed, especially for environmental samples. It could be possible that the presence of a high number of dead cells with respect to the number of viable cells, may negatively affect the performance of this technique (Pan and Breidt, 2007). Kantonale Laboratorium (2009) found that dead bacteria should not exceed live bacteria by a factor of 100 without impacting PMA-qPCR, and free DNA in copy numbers greater than 4×10^5 can mask the detection of viable cells (Kantonale Laboratorium, 2009). A possible reason to explain that is that PMA amount can be insufficient or that reaction efficiency was not enough to completely suppress the free DNA as well as the DNA from dead cells.

Although a weak correlation was found between both culture and PMA-qPCR methods for *Legionella* detection (R^2= 0.51), when all water samples are taken into account, a stronger correlation (R^2= 0.87) was observed, for 9 out of 13 determined samples. Furthermore, and most important, PMA-qPCR technique showed the same *Legionella* colonization dynamic than culture; therefore the same conclusions can be achieved but at a faster extent.

4.5. Conclusions

The results presented in this chapter show that the use of reclaimed water does not mean greater pollution if compared to well water, emphasizing the importance of that reclaimed water must be subjected to disinfection treatment prior to its use.

Irrespective of the power of disinfection for wastewater treatment, it is clearly evident that if reclaimed water requires a storage step before its use, it will be necessary an *in situ* disinfection to ensure the biological growth control during storage. Moreover, *in situ* disinfection processes allow to better face the challenges related with water quality variability.

Effective monitoring of water is critical for public health and environment protection. Real-time molecular techniques let perform a rapid screening of water quality, obtaining timely and reliable data for an effective quality control, as well as a quick response time in case of emergency. Even though qPCR cannot be a substitute for the isolation of *Legionella* by culture yet, it may be regarded as a useful monitoring tool, and as a complementary method as showed in this study. It is worth to mention that recently an ISO Technical Specification for detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* in water samples by using qPCR was published (ISO/TS 12869). This could be thought as a first step towards the standardization of this molecular technique. Additionally, the development of the viable-qPCR technique using EMA or PMA means a step forward in the field of molecular techniques. This technique is also a powerful monitoring tool that allows following disinfection process, and assessing changes in water quality.

It is important to note that the speed, specificity and sensitivity of the qPCR and PMA-qPCR techniques are prone for optimization of a number of factors that result in a good protocol. Some of these factors are the technical abilities of the lab workers, the thermal cycler performance, the pre-treatment of the sample (concentration and DNA purification), the reagents used, the gene target, and the primers and fluorogenic chemistries used. For example, for the qPCR protocol used in this study, quantification limit could have been decreased if dual-labeled fluorogenic sequence-specific probes were used instead of SYBR Green, which is a non-sequence-specific intercalating dye. This is mainly due to the fact that non-specific amplification in diluted samples is reduced when highly specific probes are used. Regarding optimization of the PMA-qPCR technique, in next Chapters of this dissertation, this viable PCR technique is applied and optimized to be used for microbial detection in environmental samples.

Although we must remember that stronger conclusions require more powerful experiments, carried out in pilot plants simulating real operation conditions of cooling towers, with larger number of samples and multiple experimental replicates, the findings of this study help to promote reuse of reclaimed water, so important nowadays where lack or lessening of water sources; and water quality deterioration represents serious concerns for many water users in various parts of the world. Moreover, the obtained results reaffirm the key role of disinfection in the use of reclaimed water. At this point, work should be done towards meeting the microbiological quality criteria required by regulations and guidelines, optimizing the WWTP tertiary treatment, as well as implementing efficient and economic disinfection processes by *in situ* treatment of reclaimed water at the end point use.

4.6. References

Ajibode, O.M., Rock, C., Bright, K., McLain, J.E.T., Gerba, C. P., Pepper, I.L. 2013. Influence of residence time of reclaimed water within distribution systems on water quality. J. Water Reuse Desalin. doi:10.2166/wrd.2013.088.

Alonso, M.C., Dionisio, L.P.C., Bosch, A., Pereira de Moura, B.S., Garcia-Rosado, E., Borrego, J.J. 2006. Microbiological quality of reclaimed water used for golf courses' irrigation. Water Sci. Technol., 54, pp. 109-117.

Association Française de Normalisation (AFNOR). 2006. AFNOR XP T90-471 Détection et quantification des *Legionella* et/ou *Legionella pneumophila* par concentration et amplification génique par reaction de polymérisation en chaîne (PCR). La Plaine Saint-Denis, France.

Alonso, J.P. 2004. Community outbreak of Legionnaires' disease related to hospital cooling towers in Zaragoza, Spain. Eurosurveillance Weekly, 8, pp. 3.

Behets, J., Declerck, P., Delaedt, Y., Creemers, B., Ollevier, F. 2007. Development and evaluation of a Taqman duplex real-time PCR quantification method for reliable enumeration of *Legionella pneumophila* in water samples. J. Microbiol. Methods, 68, pp. 137-144.

Carducci, A., Verani, M., Battistini, R. 2010. *Legionella* in industrial cooling towers: monitoring and control strategies. Lett. Appl. Microbiol., 50, pp. 24-29.

Cazurra, T. 2008. Water reuse of south Barcelona's wastewater reclamation plant. Desalination, 218, pp. 43-51.

Chang, B., Sugiyama, K., Taguri, T., Maekawa, J.A., Kura, F., Watabe, H. 2009. Specific detection of viable *Legionella* cells by combined use of photoactivated ethidium monoazide and PCR/real-time PCR. Appl. Environ. Microbiol., 75(1), pp. 147-153.

Chien, S.H., Chowdhury, I., Hsieh, M.K., Li, H., Dzombak, D.A., Vidic, R.D. 2012. Control of biological growth in recirculating cooling systems using treated secondary effluent as makeup water with monochloramine. Water Res., 46, pp. 6508-6518.

Cirelli, G.L., Consoli, S., Licciardello, F., Aiello, R., Giuffrida, F., Leonardi, C. 2012. Treated municipal wastewater reuse in vegetable production. Agr. Water Manage., 104, pp. 163-170.

CTI. 2008. Guideline: Best practices for control of *Legionella*. Cooling Technology Institute, U.S.A. In: Chien, S.H., Chowdhury, I., Hsieh, M.K., Li, H., Dzombak, D.A., Vidic, R.D. 2012. Control of biological growth in recirculating cooling systems using treated secondary effluent as makeup water with monochloramine. Water Res., 46, pp. 6508-6518.

Devos, L., Clymans, K., Boon, N., Verstraete, W. 2005. Evaluation of nested PCR assays for the detection of *Legionella pneumophila* in a wide range of aquatic samples. J. Appl. Microbiol., 99, pp. 916-925.

Qiagen. 2006. DNeasy® Blood & Tissue Kit Handbook. Protocol: purification of total DNA from animal blood or cells (spin-column protocol), pp. 25-27. Available in: http://www.qiagen.com/products/catalog/sample-technologies/dna-sample technologies/genomic-dna/dneasy-blood-and-tissue-kit#resources. Last access 10/23/2013.

Dondero, T.J., Rendtorff, R.C., Mallison, G.F., Weeks, R.M., Levy, J.S., Wong, E.W., Schaffner, W. 1980. An outbreak of Legionnaires' disease associated with a contaminated air-conditioning cooling tower. N. Engl. J. Med., 302, pp. 365-70.

Ferré, M.R., Arias, C., Oliva, J.M., Pedrol, A., Pellicer, T., Roura, P., Dominguez, A. 2009. A community outbreak of Legionnaires' disease associated with a cooling tower in Vic and Gurb, Catalonia (Spain) in 2005. Eur. J. Clin. Microbiol. Infect. Dis., 28, pp. 153-159.

Fittipaldi, M., Codony, F., Morató, J. 2010. Comparison of conventional culture and real-time quantitative PCR using SYBR Green for detection of *Legionella pneumophila* in water samples. Water SA, 36, pp. 417-424.

Garcia-Fulgueiras, A., Navarro, C., Fenoll, D., Garcia, J., Gonzalez-Diego, P., Jimenéz-Buñuales, T. 2003. Legionnaires' disease outbreak in Murcia, Spain. Emerg. Infect. Dis., 9, pp. 915-921.

Green, P.N., Pirrie, R.S., 1993. A laboratory apparatus for the generation and biocide efficacy testing of *Legionella* biofilms. J. Appl. Bacteriol., 74, pp. 388-393.

Hayden, R.T., Uhl, J.R., Qian, X., Hopkins, M.K., Aubry, M.C., Limper, A.H., Lloyd, R.V., Cockerill, F.R. 2001. Direct detection of *Legionella* species from bronchoalveolar lavage and open lung biopsy specimens: Comparison of LightCycler PCR, *in situ* hybridization, direct fluorescence antigen detection, and culture. J. Clin. Microbiol., 39, pp. 2618-2626.

Higgins, J., Warnken, J., Sherman, P.P., Teasdale, P.R. 2002. Survey of users and providers of recycled water: quality concerns and directions for applied research. Water Res., 36, pp. 5045-5056.

Hugosson, A., Hjorth, M., Bernarder, S., Claesson, B.E., Johansson, A., Larsson, H., Nolskog, P., Pap, J., Svensson, N., Ulleryd, P. 2007. A community outbreak of Legionnaires' disease from an industrial cooling tower: assessment of clinical features and diagnostic procedures. Scand. J. Infect. Dis., 3 , pp. 217-224.

Jenkins, M., Fisher, D., Endale, D. 2009. Quicker die-off of fecal indicator bacteria than *E. coli* 0157:H7 in a pond impacted by animal agriculture. American Society for Microbiology Annual Meeting, May 17-21, 2009, Philadelphia, Pennsylvania, US.

Johansen, C., Falholt, P., Gram, L., 1997. Enzymatic removal and disinfection of bacterial biofilms. Appl. Environ. Microbiol., 63 , pp. 3724-3728.

Kantonale Laboratorium. 2009. Development of a method to distinguish between DNA from dead or living microorganisms. EFBS-Project Report 04.1240.PZ/ H355-0649. http://www.efbs.admin.ch/fileadmin/efbsdateien/dokumentation/
Publikationen/Report_PMA_Project_final.pdf.

Kao, P.M., Tung, M.C., Hsu, B.M., Chiu, Y.C., She, C.Y., Shen, S.M., Huang, Y.L., Huang, W.C. 2013. Identification and quantitative detection of *Legionella* spp. in various aquatic environments by real-time PCR assay. Environ. Sci. Pollut. Res., 10.1007/s11356-013-1534-z.

Kim, B.R., Anderson, J.E., Mueller, S.A., Gaines, W.A, Kendall, A.M., 2002. Literature review-efficacy of various desinfectants against *Legionella* in water systems. Water Res., 36, pp. 4433-4444.

Kohli, A., Frenken, K. 2011. Cooling water for energy generation and its effects on national-level water statistics. AQUASTAT Programme of FAO, www.fao.org.

Kool, L.J. 2002. Control of *Legionella* in drinking water systems: impact of monochloramine, *Legionella*. In: Marre, R., Kwaik, Y.A., Bartlett, C., Cianciotto, N.P., Fields, B.S, Frosch, M., Hacker, J., Lück, P.S. (Eds.), *Legionella*. ASM Press, Washington, DC, pp.411-418, 1-55581-230-9.

International Standard Organization. 1998. ISO 11731. Water quality – Detection and enumeration of *Legionella*. Geneva, Switzerland.

International Standard Organization. 2012. ISO/TS 12869. Water quality — Detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR). Geneva, Switzerland.

Lehtola, M.J., Torvinen, E., Kusnetsov, J., Pitkänen, T., Maunula, L., von Bonsdorff, C.H., Martikainen, P.J., Wilks, S.A., Keevil, C.W., Miettinen, I.T. 2007. Survival of *Mycobacterium avium, Legionella pneumophila*, *Escherichia coli*, and Caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. Appl. Environ. Microbiol., 73, pp. 2854-2859.

Li, H., Chien, S.H., Hsieh, M.K., Dzombak, D.A., Vidic, R.D. 2011. Escalating water demand for energy production and the potential for use of treated municipal wastewater. Environ. Sci. Technol., 45, pp. 4195-4200.

Lin, Y.S., Stout, J.E., Yu, V.L., Vidic, R.D. 1998. Disinfection of water distribution systems for *Legionella*. Semin. Respir. Infect., 13, pp. 147–159.

Ludensky, M. 2005. Microbiological control in cooling water systems. In: Paulus, W. (Ed.). 2005. Directory of Microbiocides for the Protection of Materials: A Handbook. Springer Verlag. Germany, pp. 121-139.

McFeters, G.A., Bissonnette, G.K., Jezeski, J.J., Thomson, C.A., Stuart, D.G. 1974. Comparative survival of indicator bacteria and enteric pathogens in well water. Appl. Environ. Microbiol., 27, pp. 823-829.

Metcalf & Eddy Inc., Tchobanoglous, G. 1991. Wastewater Engineering, Treatment, Disposal and Reuse. McGraw-Hill International. Singapore.

Momba, M.N.B, Binda, M.A. 2002. Combining chlorination and chloramination processes for the inhibition of biofilm formation in drinking surface water system models. J. Appl. Microbiol., 92, pp. 641-648.

Muraca, P., Stout, J.E., Yu, V.L. 1987 Comparative assessment of chlorine, heat, ozone, and UV light for killing *Legionella pneumophila* within a model plumbing systems. Appl. Environ. Microbiol., 53, pp. 447–453.

Nocker, A., Cheung, C.Y., Camper, A.K., 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead. cells. J. Microbiol. Methods, 67, pp. 310-320.

Nocker, A., Sossa, K.E., Camper, A.K. 2007. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. J. Microbiol. Methods, 70, pp. 252–260.

Oguma, K., Katayama, H., Ohgaki, S. 2004. Photoreactivation of *Legionella pneumophila* after inactivation by low-or medium-pressure ultraviolet lamp. Water Res., 38, pp. 2757-2763.

Palese, A.M., Pasquale, V., Celano, G., Figliuoloc, G., Masid, S., Xiloyannisa, C. 2009. Irrigation of olive groves in Southern Italy with treated municipal wastewater: effects on microbiological quality of soil and fruits. Agr. Ecosyst. Environ., 129, pp. 43-51.

Pan, Y., Breidt, F. 2007. Enumeration of viable *Listeria monocytogenes* cells by real time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. Appl. Environ. Microbiol. 73, pp. 8028–8031.

Pankhurst, C.L., Philpott-Howard, J.N., Hewitt, J.H., Casewell, M.W. 1990. The efficacy of chlorination and filtration in the control and eradication of *Legionella* from dental chair water systems. J. Hosp. Infect., 16, pp. 9-18.

Pastoris, M.C., Ciceroni, L., Monaco, R.L., Goldoni, P., Mentore, B., Flego, G., Cattani, L., Cuarrocchi, S., Pinto, A., Visca, P. 1997. Molecular epidemiology of an outbreak of Legionnaires' disease associated with a cooling tower in Genova-Sestri Ponente, Italy. Eur. J. Clin. Microbiol. Infect. Dis., 16, pp. 883-892.

Pedrero, F., Alarcón, J.J. 2009. Effect of treated wastewater irrigation on lemon trees. Desalination, 246, pp. 631-639.

Pedrero, F., Kalavrouziotis, I., Alarcón, J.J., Koukoulakis, P., Asano, T. 2010. Use of treated municipal wastewater in irrigated agriculture-Review of some practices in Spain and Greece. Agr. Water Manage., 97, pp. 1233-1241.

Qureshi, B.A., Zubair, S.M. 2006. A complete model of wet cooling towers with fouling in fills. Appl. Therm. Eng., 26, pp. 1982-1989.

Rasmussen, R. 2001. Quantification on the LightCycler. In: Meuer, S., Witwe, C., Nakagawara, K. (Eds.) Rapid Cycler Real-Time PCR, Methods and Applications. Springer, Berlin. Germany, pp. 21-34.

Rebhun, M., Engel, G. 1988. Reuse of wastewater for industrial cooling systems. J. Water Pollut. Control Fed., 60, pp. 237-241.

Rebhun, M., Grynberg, H. 1985. Use of wastewater for cooling systems. In: Rebhun, M., Engel, G. 1988. Reuse of wastewater for industrial cooling systems. J. Water Pollut. Control Fed., 60, pp. 237-241.

Royal Decree-Law 140/2003 of health quality criteria for drinking water. 2003. Official Bulletin of State, BOE 45.

Royal Decree-Law 1620/2007 of treated wastewater reuse. 2007. Official Bulletin of State, BOE, 294.

Rudi, K., Moen, B., Dromtorp, S.M., Holck, A.L. 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl. Environ. Microbiol., 71, pp. 1018-1024.

Sabria, M., Alvarez, J., Dominguez, A., Pedrol, A., Sauca, G., Salleras, L., Lopez, A., Garcia-Nuñez, M.A., Parron, I., Barrufet, M.P. 2006. A community outbreak of Legionnaires' disease: evidence of a cooling tower as the source. Clin. Microbiol. Infect., 12 , pp. 642-647.

Standard Methods for the Examination of Water and Wastewater. 1999. American Public Health Association, American Water Works Association, Water Environment Federation. US.

Touron-Bodilis, A., Pougnard, C., Frenkiel-Lebossé, H., Hallier-Soulier, S. 2011. Usefulness of real-time PCR as a complementary tool to the monitoring of *Legionella* spp. and *Legionella pneumophila* by culture in industrial cooling systems. J. Appl. Microbiol., 111, pp. 499-510.

Türetgen, I., Cotuk, A. 2007. Monitoring of biofilm-associated *Legionella pneumophila* on different substrata in model cooling tower system. Environ. Monit. Assess, 125, pp. 271-279.

Varma, M., Field, R., Stinson, M., Rukovets, B., Wymer, L., Haugland, R. 2009. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. Water Res., 43, pp. 4790-4801.

Wada, Y., van Beek, L.P.H., Bierkens, M.F.P. 2011. Modelling global water stress of the recent past: on the relative importance of trends in water demand and climate variability. Hydrol. Earth Syst. Sci., 15, pp. 3785–3808.

Wahman, D.G., Wulfeck-Kleier, K.A., Pressman, J.G. 2009. Monochloramine disinfection kinetics of *Nitrosomonas europaea* by propidium monoazide quantitative PCR and live/dead BacLight methods. Appl. Environ. Microbiol., 75, pp. 5555-5562.

Wellinghausen, N., Frost, C., Marre, R. 2001. Detection of legionellae in hospital in water samples by quantitative real-time LightCycler PCR. Appl. Environ. Microbiol. 67, pp. 2047-2053.

Wijesinghe, B., Kaye, R.B., Fell, C. J. D. 1996. Reuse of treated sewage effluent for cooling water make up: a feasibility study and a pilot plant study. Wat. Sci. Tech., 33, pp. 363-396.

World Bank. 2001. World Development Indicator (WDI). In UNESCO. 2003. Water for People, Water for Life, United Nations World Water Development Report. Executive Summary, pp. 19. www.unesdoc.unesco.org

Wright, J.B, Ruseska, I., Consterton, J.W., 1991. Decreased biocide susceptibility of adherent *Legionella pneumophila*. J. Appl. Bacteriol., 71, pp. 531-538.

Yañez, M.A., Carrasco-Serrano, Barberá, V.M., Catalán, V. 2005. Quantitative detection of *Legionella pneumophila* in water samples by immunomagnetic purification and real-time PCR amplification of the *dotA* gene. Appl. Environ. Microbiol., 71 , pp. 3433-3441.

Yañez, M.A., Nocker, A., Soria-Soria, E., Múrtula, R., Martínez, L., Catalán, V. 2011. Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. J. Microbiol. Methods, 85, pp. 124-130.

Chapter 5

Difficulties and optimization strategies in the quantification of live *Legionella pneumophila* by viable qPCR in the presence of dead cells *

One of the main limitations of DNA-based microbial diagnosis methods, including PCR, is the inability to discriminate between viable and non-viable cells. Herein, the ability of real-time PCR (qPCR) combined with propidium monoazide (PMA) pre-treatment to quantify live *Legionella pneumophila* in water samples in the presence of dead cells has been evaluated. Defined proportions of live and dead cells were exposed to PMA, and *Legionella pneumophila* levels were determined by qPCR. Our results showed the limits of viable qPCR in the presence of high levels of dead cells, because the qPCR signal is not suppressed entirely and false-positive results can be obtained. Thus, the viable qPCR method, by itself without additional improvements, may not be suitable for the correct quantification of *Legionella pneumophila* in environmental samples with a high number of dead cells, and/or high contaminated samples exposed to disinfection treatments. Complementary strategies to avoid false-positive detection are discussed.

*To be published in Applied Microbiology and Biotechnology as:
Fittipaldi, M., Rajal, V., Codony, F., Morató, J. Difficulties in the quantification of live *Legionella pneumophila* by viable qPCR in presence of dead cells.

5.1. Introduction

Legionella pneumophila, commonly found in aquatic environments, is the most important bacterium of the *Legionella* genus because of its high impact upon public health (Gomez Valero et al., 2009; Yu et al., 2002). *Legionella* are difficult to control in environmental sources due to association with biofilms (Chaabna et al., 2013), parasitism of protozoa hosts (Codony et al., 2012), and consequently their resistance to disinfectants. There exist, in most developed countries, guidelines for controlling the growth of environmental *Legionella.* A risk assessment and management approach is considered by the American Society of Heating, Refrigerating and Air conditioning Engineers (ASHRAE) and the UK Health and Safety Executive (Mascone, 2008).

Nowadays there are several methods for *Legionella* detection and enumeration, however, conventional culture technique is still the standard method for the detection and quantification of *Legionella* in water samples (Yaradou et al., 2007), although the test based on combined magnetic immunocapture and enzyme-immunoassay developed by Biotica company is a certified method for the fast detection of *Legionella pneumophila* in water samples by the AOAC Research Institute[1]. Moreover, as it was mentioned in Chapter 4 of this dissertation, an ISO Technical Specification for detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* in water samples by using qPCR has recently been published (ISO/TS 12869).

It is important to note that *Legionella* culture technique does have some limitations. This method is time consuming, given that it requires long incubation periods. The presence of other microorganisms may interfere with *Legionella* growth and complicate the interpretation of culture results. There is the possibility of bacterial loss during the decontamination step with heat or acid. Moreover, *Legionella* can be present as viable but non-culturable cells (VBNC) (Hussong et al., 1987), therefore precluding its detection by culture-based methods. To avoid all these disadvantages, several rapid and sensitive PCR-based methods for the detection of *Legionella pneumophila* in clinical and environmental samples have been described (Ballard et al., 2000; Behets et al., 2007; Joly et al., 2006; Levi et al., 2003; Mérault et al., 2011; Wellinghausen et al., 2001; Wilson et al., 2003).

The application of PCR for the detection of microorganisms is becoming a widely used practice for environmental, food and clinical samples. Conventional PCR followed by electrophoresis in agarose gels is commonly used for testing the presence or absence of pathogens. However, quantitative PCR is shifting this approach since it offers the possibility of quantifying the contamination. Numerous tests for qPCR detection have already been designed and validated using different chemistries, either with intercalating agents (Fittipaldi et al., 2010a; Kao et al., 2013) or with fluorescent probes (Diederen et al., 2007; Wellinghausen et al., 2001). Real-time PCR offer the benefit of speed over traditional culturing methods and viable non-culturable cells may be detected for these (Bej et al., 1991). However, the main inconvenience of DNA-based microbial diagnosis methods, including PCR, is the inability to discriminate between viable and non-viable cells because DNA persists in the environment after cells have lost viability in the range of several days to three weeks (Josephson et al., 1993; Masters et al., 1994). So, DNA-based quantification methods use to overestimate the number of live cells and, therefore the sanitary risk associated to such large figure.

Over the last years, it has been showed that certain nucleic acid-binding dyes can selectively enter cells with compromised cell membrane integrity and subsequently be covalently linked to DNA upon light exposure. Ethidium monoazide (EMA) and propidium monoazide (PMA) are the two commonly used DNA intercalating dyes that can enter membrane-injured cells and crosslink to DNA by photoactivation (Nocker et al., 2006; Nogva et al., 2003). If DNA is present in this bound state, it cannot theoretically be amplified by PCR (Chang et al., 2009; Rudi et al., 2005; Soejima et al., 2007). The method's underlying principle is based on membrane integrity as the viability criterion. This hypothesis could be a limitation for its

[1] http://www.biotica.es/es. Last access 09/17/2013

applicability (Girones et al., 2010). Nevertheless, cells that maintain membrane integrity and retain some metabolic activity or responsiveness are currently considered viable (Keer and Birch, 2003). Recently, these viability (v-PCR) methods have been evaluated using several microorganisms including bacteria (Agusti et al., 2010; Bae and Wuertz, 2009; Cawthorn and Witthuhn, 2008; Delgado Viscogliosi et al., 2009; Elizaquível et al., 2013, Pan and Breidt, 2007; Rudi et al., 2005; Vendrame et al., 2013; Yasunaga et al., 2013), bacterial spores (Rawsthorne et al., 2009), viruses (Fittipaldi et al., 2010b; Graiver et al., 2010; Sanchez et al., 2012), yeast (Agustí et al., 2013; Andorrà et al., 2010; Shi et al., 2012), fungi (Blooi et al., 2013; Crespo-Sempere et al., 2013; Vesper et al., 2008), and protozoa (Brescia et al., 2009; Fittipaldi et al., 2011). These methods are promising for DNA-based differentiation between viable and non-viable bacteria, therefore, they are enjoying an increasing popularity in diverse areas including *Legionella* monitoring in water systems (Chen and Chang, 2010; Delgado Viscogliosi et al., 2009; Qin et al., 2012; Yañez et al., 2011). However, the method also presents some limitations, especially when complex environmental samples, like wastewater, are analyzed (Wagner et al., 2008). In some cases, intact membrane cells have showed to be susceptible to dye uptake, mainly when EMA is used, leading to false-negative signals (Kobayashi et al. 2009; Nocker and Camper, 2006). This can be overcome by optimizing dye concentrations (Meng et al., 2010), modifying incubation time and temperature (Flekna et al., 2007; Soejima et al., 2011), or by using PMA instead of EMA depending on the matrix sample and the microorganisms to analyze (Fittipaldi et al., 2012; Nocker et al., 2006). On the other hand, the incomplete exclusion of dead cell amplification signals (Bae and Wuertz, 2009; Chang et al., 2010; Fittipaldi et al., 2010b; Kralik et al., 2010) leading to false-positive signals has been reported as a main drawback of the v-PCR technique (Nkuipou-Kenfack et al., 2013). Many factors could cause bias in the application of PMA-qPCR, for example the presence of a high number of dead cells is one possible reason for false-positive detection signals (Wang et al., 2009).

As water is a complex matrix with a mixture of live and dead cells, the aim of this study was to evaluate the possible effect of the presence of dead cells on the quantification of live cells. In this chapter, the methodology for PMA treatment coupled with qPCR (v-qPCR) to quantify live cells of *Legionella pneumophila* in presence of dead cells is thoroughly discussed.,

5.2. Materials and Methods

5.2.1. Microorganism and medium

Legionella pneumophila serogroup 1 (NCTC12821) was cultured in *Legionella* selective medium GVPC (Oxoid, Hampshire, UK) for 4 days at 37 ºC. Once the culture was ready, a bacterial suspension was prepared by transferring single colonies into a tube with sterile 1X phosphate-buffered saline solution (PBS) at pH 7.4. The optical density at 600 nm (OD_{600}) was adjusted to 0.2, which approximately corresponds to a concentration of 10^8 colony forming unit (CFU) per mL, as confirmed by plate count. This suspension was considered as the original stock of live cells. Serial ten-fold dilutions were prepared from the suspension, using sterile PBS to obtain the set of dilutions of live cells that was later used for experiments.

5.2.2. Killing conditions

The original stock of dead cells was obtained by heat-killing the cells from original stock of live cells for 10 minutes at 90 ºC. Loss of culturability was verified by streaking 200 µL of cell suspension and dilutions on GVPC agar plates (Oxoid, Hampshire, Oxoid) followed by incubation at 37 ºC for 10 days and the presence of live cells also was verified by using Live-Dead® BacLight TM Bacterial Viability Kit (Life Technologies S.A, Madrid, Spain).

Serial ten-fold dilutions were prepared from the stock of dead cells using sterile PBS to obtain the set of dilutions of dead cells that was later used for the experiments.

5.2.3. PMA treatment

PMA (Biotium, Inc., Hayward, USA) was dissolved in a 20% dimethylsulfoxide (Sigma Aldrich, Madrid, Spain) and 80% PCR grade water (Eppendorf, Madrid, Spain) solution, to obtain a 2 mM PMA stock solution. Briefly, 487.5 µL of the bacteria suspension was placed in a propylene Eppendorf 1.5 mL tube and 12.5 µL of PMA stock solution was rapidly added in a darkened room to obtain a final PMA concentration of 50 µM. The resultant cell suspension was incubated for 5 min in the dark at room temperature to allow PMA to enter into the cells with compromised or damaged membranes. The samples were then placed in an ice bath to avoid overheating and exposed for 2 min to a 650 W halogen light source (Quartzline Halogen Lamp, General Electric Company, Ohio, US) placed 30 cm from the sample tubes. Shorter distances overheated the samples, and in some cases broke down the microtubes and the sample was lost. After cross-linking photo-induction, cells were pelleted in a minicentrifuge (miniSpin plus, Eppendorf, Hamburg, Germany) at 14500 rpm for 5 min, the supernatant was discarded, and the pellet was resuspended in 200 µL of PBS.

5.2.4. Nucleic acid extraction

DNA was extracted from 200 µL samples using the EZNA tissue DNA purification kit 1×200 (OMEGA Bio-Tek, Norcross, US) following the manufacturer's instructions for cultured cells with a two-step 100 µL final elution. The final eluate was used for qPCR determination. The same extraction protocol was followed for all of the bacterial dilutions, live cells and dead cells, without PMA treatment and also for the resuspended pellet after PMA treatment.

5.2.5. Quantitative PCR

The quantification of *Legionella pneumophila* was performed by qPCR using a LightCycler 1.5 PCR system (Roche Molecular Diagnostics, Mannheim, Germany) and a previously optimized protocol (Fittipaldi et al., 2010a). The reaction mixture, 20 µL total, was composed of 10 µL SYBR Green (Quantifast SYBR Green PCR kit, Qiagen, Hilden, Germany), 10 µL of sample, 0.5 µM final concentration of *mip* reverse (LpmipAr, AACGCCTGGCTTGTTTTTGT) and forward (LpmipAf, ACCGAACAGCAAATGAAAGA) primers yielding a 144 bp product (Hayden et al., 2001), and 0.2 U of uracil-DNA-glycosylase (UDG, New England BioLabs, Suffolk, UK).

The experimental protocol consisted of one step of 2 min at 50 ºC to allow UDG to break down the possible contaminating amplicons, one step of 5 min at 95 ºC for Taq polymerase activation, 45 cycles of PCR amplification (95 ºC for 10 s, and 60 ºC for 30 s), a melting temperature ramp from 65 to 95 ºC at 0.1 ºC per second, and a final cooling step from 95 ºC to 40 ºC.

All the measurements were performed in duplicate. A positive control consisting of *Legionella pneumophila* DNA and a negative control with PCR grade water, instead of template, were included with every determination to verify the proper functioning of the amplification-quantification system and the absence of cross-contamination, respectively.

5.2.6. Experiments

Two standard curves were constructed using the ten-fold serial dilutions (10^8 to 10^4) of the eluate from original stock of live cells and original stock of dead cells nucleic acid extraction after PMA pretreatment to determine by comparison whether dead cells with PMA amplified correctly. Assays were performed by triplicate.

To determine the possible effects of the presence of dead cells on the quantification of live cells by PMA pretreatment and qPCR, a set of experiments with different concentrations of

live and dead cells were prepared. Volumes of 500 µL containing respectively a final concentration of 10^7, 10^5, and 10^3 dead cells per mL, were prepared containing final concentrations of 10^7, 10^6, 10^5, 10^4, 10^3 live cells per mL (15 combinations in total) according to the experimental design depicted on Table 5.1 . A determined volume of each stock suspension (50 µL) was mixed with a determined PBS volume (387.5 µL) and 12.5 µL of PMA (final concentration 50 µM). The prepared samples were incubated for 5 min at room temperature with occasional mixing and photoactivated. The total volume sample was 500 µL. These bacterial suspensions were treated with PMA, the nucleic acid was extracted, and qPCR was performed according to the procedures described above. Assays were performed by duplicate.

Table 5.1. Experimental design to study the effect of the presence of dead cells in the detection of *Legionella pneumophila* live cells.

No	Live Stock (CFU/mL)	Dead Stock (CFU/mL)	Live Mix (CFU/mL)	Dead Mix (CFU/mL)
1	10^8	10^8	10^7	10^7
2	10^7	10^8	10^6	10^7
3	10^6	10^8	10^5	10^7
4	10^5	10^8	10^4	10^7
5	10^4	10^8	10^3	10^7
6	10^8	10^6	10^7	10^5
7	10^7	10^6	10^6	10^5
8	10^6	10^6	10^5	10^5
9	10^5	10^6	10^4	10^5
10	10^4	10^6	10^3	10^5
11	10^8	10^4	10^7	10^3
12	10^7	10^4	10^6	10^3
13	10^6	10^4	10^5	10^3
14	10^5	10^4	10^4	10^3
15	10^4	10^4	10^3	10^3

Note. Live and dead cell suspension stocks were prepared at different concentrations (Live and Dead Stock). Fifty microliters of each stock (Live or Dead) were used to prepare 500 µL of Mix.

5.2.7. Differential stain of live and dead cells on original stock of live cells and original stock of dead cells

LIVE/DEAD® BacLight TM Bacterial Viability Kit (Life Technologies S.A, Madrid, Spain)was used in order to differentially stain cells. This kit contains a mixture of two dyes: SYTO® 9 green-fluorescent nucleic acid stain, that labels all bacteria in a population (with intact and also with damaged membranes) and the red-fluorescent nucleic acid stain, propidium iodide, that penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9® stain fluorescence when both dyes are present. The culture and the bacterial suspensions were prepared following the manufacturer’s instructions.

A fraction of the original stock of live cells was stained in order to determine the proportion of dead or live cells. The observations were performed at 450-500 nm in a Nikon Fluorescence Microscope using an epifluorescence adapter (Nikon DM510; Duesseldorf, Germany) and a long-pass filter (B-2A, Duesseldorf, Germany).

Suspensions containing 0, 20, 40, 50, 60, 80, and 100% from original stock of live cells and original stock of dead cells were stained using the kit and following the manufacturer's procedure for Fluorescence Microplate Reader. Mixtures of 100, 90, 70, 50, 30, 10, and 0% of original stock of live cells with 0, 10, 30, 50, 70, 90, and 100 % of original stock of dead cells, respectively, were also analyzed to determine whether the presence of dead cells affected on the determination of the percentage of live cells in a mixture. The Green emission (G, all the cells: live plus dead cells) was measured at 535 nm, while the Red emission (R, only dead cells) was read at 630 nm using a Microplate reader Genios, software Magellan standard (Tecan, Meilen, Switzerland). The fluorescence ratio G/R was calculated for each sample.

5.3. Results

Standard curves were performed with ten-fold serial dilutions of live cells and dead cells suspensions with and without PMA treatment. The behavior for live cell samples with and without PMA and for dead cells without PMA was very similar as expected (Figure 5.1). The mean reaction efficiencies (± the standard deviation) for live cell samples with and without PMA treatment were 94.4%±7 and 89%±8.2 respectively; while for dead cell samples with and without PMA treatment the efficiencies were 194.7%±50.6 and 87.7%±2.9, respectively. The correlation coefficients were larger than 0.99 for live cells with and without PMA, and also for dead cells without PMA treatment. Therefore, PMA treatment seems to not affect the detection of live cells.

As shown in Figure 5.1, targets from dead cells with PMA pretreatment amplified even when they were not supposed to. Consequently, false-positive results were observed. However, it is worth of mention that when 500 µL of dead cell suspension with concentration of 10^4 CFU/mL was treated with PMA the DNA amplification was completely suppressed. These results clearly did not fit a straight line. Interestingly, amplification signal reduction for dead cells was in the range of 2 to 3.7 log units. This could indicate that the used PMA treatment has limited capacity, maybe determined by the stoichiometry, to block the DNA amplification from dead cells, independently of the number of dead cells present in the sample, at least in the type of cell suspension analyzed.

The results presented above (Figure 5.1) correspond to all live or all dead cells from a pure culture and are not representative of an environmental sample where a mixture of live and dead cells would most likely be found. Mixtures consisting of different concentrations of live and dead cells were studied as model solutions of real environmental samples, to determine whether it was possible to differentiate such mixtures by the application of PMA and qPCR. For the same concentration of live cells, different Ct values were obtained depending on the concentration of damaged-membrane cells present in the sample (Figure 5.2). The results showed that in mixtures containing more than 10^5 CFU/mL of dead cells false-positive results are obtained. Thus, v-qPCR method may overestimate the number of intact membrane cells in samples. When the ratio of dead cells to live cells was 10^2 or higher the relationship between the number of *Legionella pneumophila* cells and the Ct values was affected, especially when the concentration of dead cells was higher than 10^5 CFU/mL (Figure 5.3).

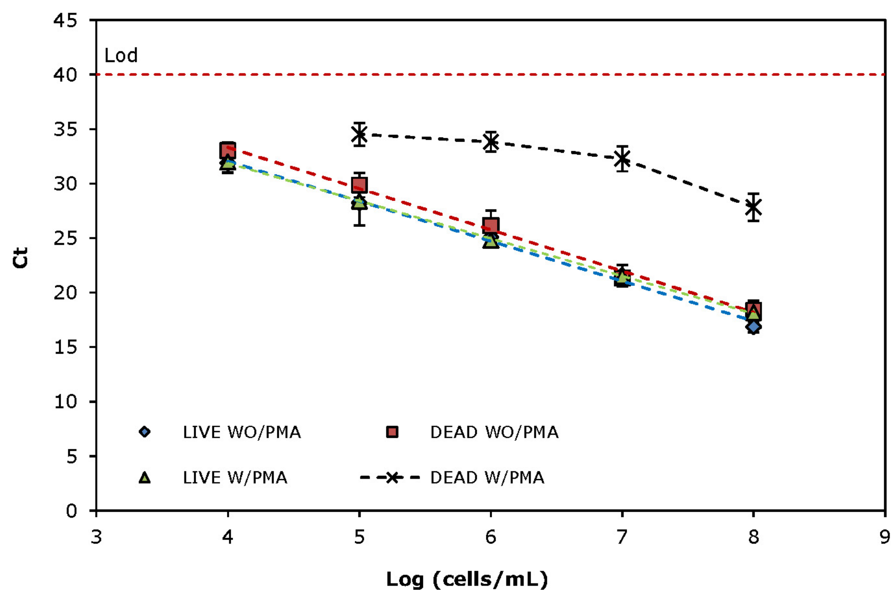


Figure 5.1. Standard curves for *Legionella pneumophila* live and dead cells with and without 50 µM PMA treatment. Dead cells were obtained by exposing them to 90 ºC for 10 min. Each point corresponds to the mean of the Ct values. The error bars represent the standard deviation for three independent experiments. The limit of detection (LOD) was 50 cell/mL. WO/PMA: without PMA treatment; W/PMA: with PMA treatment.

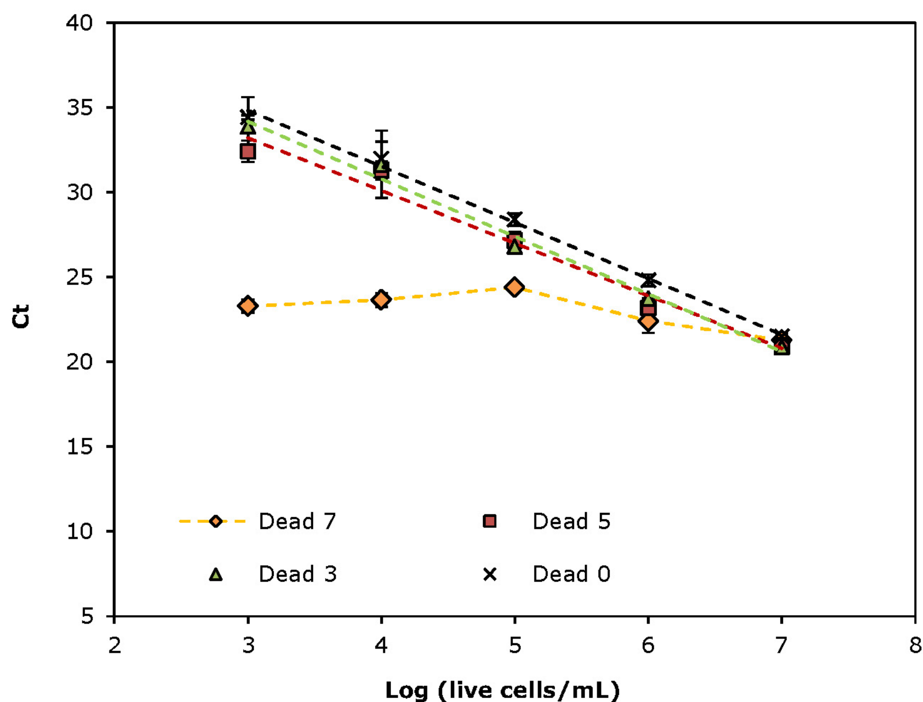


Figure 5.2. Effect of the presence of dead cells on the quantitative detection of live cells from a lived/dead mixed sample. Known *Legionella pneumophila* live cells concentrations were mixed with final known concentrations of *Legionella pneumophila* dead cells: 0 (Dead 0, no dead cells), 103 (Dead 3), 105 (Dead 5), and 107 (Dead 7) CFU/mL. Each point corresponds to the mean of Ct values. The error bars represent the standard deviation for two independent assays where each point was analyzed by duplicate.

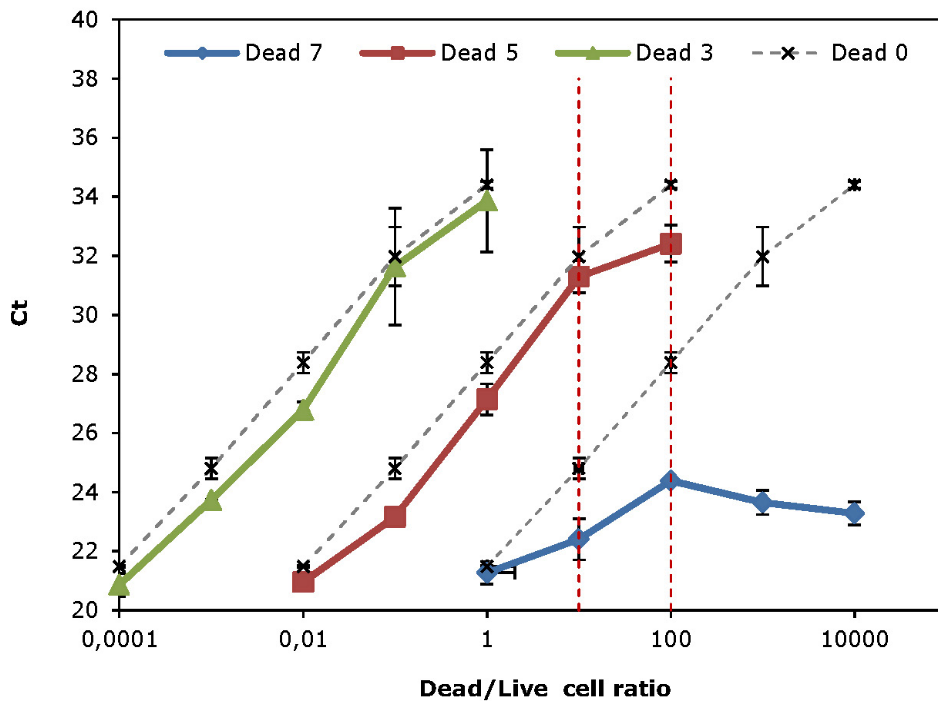


Figure 5.3. Influence of dead *Legionella pneumophila* cells in the quantification of live *Legionella pneumophila* cells. Mixtures of a fixed number of dead cells (10^7 (Dead 7), 10^5 (Dead 5), 10^3 (Dead 3), 0 (Dead 0) CFU/mL) with increasing number of live cells (10^3 to 10^7 CFU/mL) were prepared. Each point corresponds to the mean of Ct values. The error bars represent the standard deviation for two independent assays where each point was analyzed by duplicate. Red dashed vertical lines indicate possible critical Dead/Live cell ratio.

Both, slope and correlation coefficient values (Table 5.2) are negatively affected when a high concentration of dead cells, such as 10^7 CFU/mL, is present in the mixture. For the curve corresponding to this dead cell concentration, the correlation coefficient improves from 0.481 to 0.976 and the slope changes from -0.5 to -1.56 if the two last points are not considered. In the case of the Dead cells 10^5 curve, the slope changes from -3.01 to -3.49, and the correlation coefficient improves from 0,973 to 0.983 if the last point is not taken into account in the calculations.

A curious observation is, however, the fact that Ct is a linear function of the logarithm of the concentration of total cells (live plus dead) as shown in Figure 5.4, where all the combinations of live and dead cell concentrations are represented. The efficiency of amplification, related to the slope of the straight line, is similar to that obtained for different live cells alone (without dead cells) or for the mixed of different concentrations of live cells with a final dead cell concentration of 10^3 CFU/mL. This result confirms that in presence of a high number of dead cells, v-qPCR technique overestimate the live cell number present in a live-dead cell mixture due to false-positive results.

Ten percent of the original stock of live cells was determined to be damaged cells by fluorescent microscopy using the LIVE/DEAD® BacLight TM Bacterial Viability Kit. On the other hand, the fluorescence ratio G/R obtained from the fluorescence microplate reader, when graphed against the proportion of viable cells (Figure 5.5), represents the independent measurement needed to quantify the amount of viable cells contained in a mixture of live and dead cells. Although the ratio G/R increases proportionally to the concentration of dead cells (for pure dead cells suspensions) it does not affect the ratio G/R in mixtures of live and dead cells. This was verified by linearly fitting these data together with those corresponding to only live cells (R^2 = 0.959).

Table 5.2. Parameters of the correlation obtained in the study of the effect of the presence of dead cells on the quantitative detection of *Legionella pneumophila* live cells (10^3 to 10^7 CFU/mL).

Parameter	Concentration of dead cells			
	0 CFU/mL	**10^3 CFU/mL**	**10^5 CFU/mL**	**10^7 CFU/mL**
Slope	-3.32	-3.39	-3.01	-0.53
Correlation coefficient	0.998	0.988	0.973	0.481

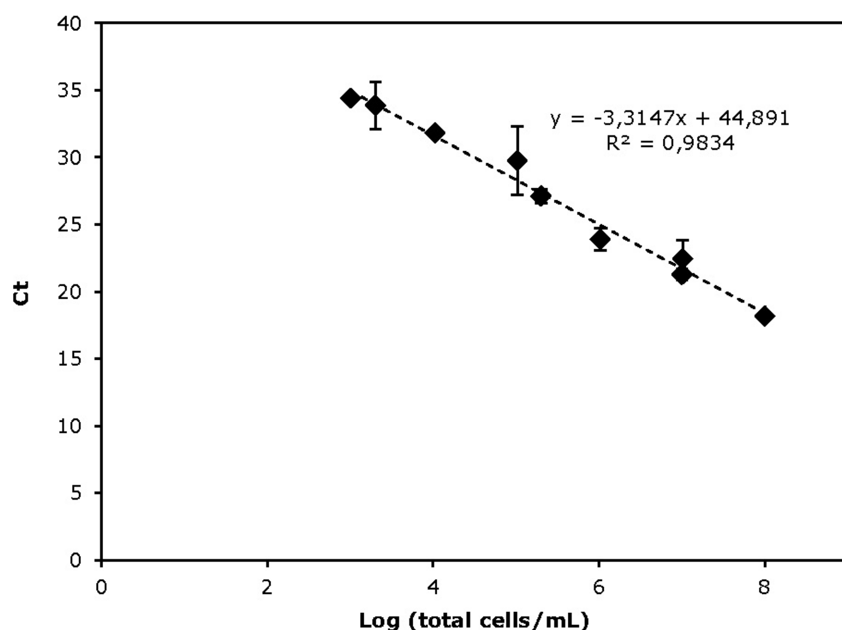


Figure 5.4. Concentration of total cells, live and dead, detected by v-qPCR from a mixture of known live and dead cell concentrations.

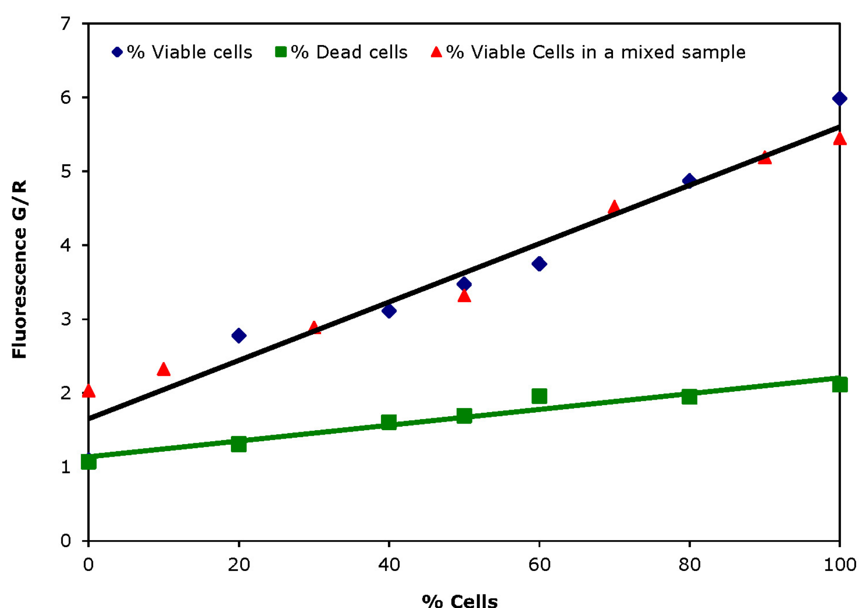


Figure 5.5. Effect of dead cell concentration on the quantitative detection of *Legionella pneumophila* live cells by fluorescence using LIVE/DEAD® BacLight TM Bacterial Viability Kit. Fluorescence ratio, Green/Red (G/R), for suspensions of live cells, dead cells, and mixtures of live and dead cells in different proportions.

5.4. Discussion

Although the v-PCR method is theoretically able to distinguish between live and dead cells when samples are treated with PMA prior to qPCR, in the different experiments performed in this work, suppression of dead cells signals was no complete, leading to an overestimation of the concentration of live cells. This fact was pointed out as one of the most eminent challenges of the v-PCR technique (Nkuipou-Kenfack et al., 2013).

The analysis of the standard curves when live and dead cells of *Legionella pneumophila* were treated or not with PMA showed that, under the studied conditions, dead cells can still produce a positive signal when they are pretreated with 50 µM PMA solution. However, as expected, a non-acceptable reaction efficiency was observed (larger than 110%) in this case. For live cells, signal reduction was moderate, being -1.3 the maximum reduction obtained in the Ct value. The presence of some cells with their membrane damaged in the live cell suspension stock can explain the little signal reduction observed for PMA treated live cells. So, under the conditions chosen, PMA does not seem to penetrate *Legionella pneumophila* live cells. The signal reduction obtained by subtracting the Ct values of PMA-treated dead cells from the Ct values of PMA-treated live cells was approximately -9.6±0.8 for all dilutions, except for the 10^5 CFU/mL which was -6.1. These results could indicate that under the studied conditions the signal reduction for dead cells was around 3 log units with independency of the cell concentration. Delgado Viscogliosi et al. (2009) observed similar results working with 2.5 µg/mL of EMA (approximately 6 µM) and heat killed *Legionella* cells. Maximum signal reductions of 4 to 5 log units were reported in many studies applying viability dyes to *Legionella* pure cultures subjected to a heat treatment (Chang et al., 2009; Chang et al., 2010; Chen and Chang, 2010; Qin et al., 2012, Yañez et al., 2011). Slimani et al. (2012) used a double PMA treatment to obtain a dead cells amplification inhibition of 3.9 log units when 6.25 µM PMA treatment was applied directly on membrane filter for the determination of VBNC *Legionella* cells.

EMA showed higher capacity than PMA to penetrate in heat-damaged *Legionella* cells, and to minimize false-positive signals (Chang et al., 2010; Chen and Chang, 2010; Yañez et al., 2011). However, it seems to affect negatively DNA amplification from live cells at certain concentrations (Chang et al., 2009; Chen and Chang, 2010; Delgado Viscogliosi et al., 2009). Therefore, to optimize the dye concentration to use in *Legionella* v-PCR is critical to reach the maximum discrimination between live and dead cells. Comparing the same dye concentration, Chang et al. (2010) found that the maximal signal reduction with killed cells was 0.5 to 1 log units higher for EMA than for PMA. These results can be explained by the higher capacity of EMA to penetrate cell membranes, but also the dye-cell incubation temperature could have played an important role. In this case, cells were maintained at 4ºC for 5 min after adding EMA or PMA at different concentrations (Chang, et al., 2010). This strategy could be useful for minimizing EMA uptake by live cells, but also for minimizing the PMA uptake by dead cells. Recently, a study performed using PMA and *Salmonella enterica* serovar *Typhimurium* and *Listeria monocytogenes* concluded that higher temperatures might be more appropriate for PMA to achieve a more efficient exclusion of dead cells signals (Nkuipou-Kenfack et al., 2013).

Apart from the dye concentration and dye incubation conditions, it has been demonstrated that the PCR product length affect the exclusion of dead cells amplification signal when the v-PCR method is applied (Banihashemi et al., 2012; Martin et al., 2013; Soejima et al., 2008; Soejima et al., 2011). For *Legionella* dead cells, signal reductions of 4 to 5 and 1.5 to 2.5 log units where reached, depending on whether qPCR was based on the amplification of 16SrRNA (454 bp) and 5SrRNA (108 bp), respectively (Chang et al., 2010). Herein a qPCR product of 144 bp was used, so higher inhibition than 3 log units could be obtained by using a longer gene target. Photoactivation step by using halogen lamps showed to be problematic. Although these lamps are functional for research purposes, excessive sample heating was observed several times. Moreover, different photoactivation efficiencies can be reached when halogen lamp are used because of the manual procedure. So the use of light-emitting diodes

(LEDs), which were first introduced for this application by Vesper et al. (2008), are a good alternative (Fittipaldi et al., 2012).

A proper method for quantification of live cells should not be influenced, or the influence must be negligible or quantifiable, by the presence of dead cells. This is not the case for the herein used PMA-qPCR method when applied to the quantitative detection of *Legionella pneumophila* since the Ct value seems to be impacted by the presence of high concentration of dead cells (Figure 5.3). For a dead cell concentration $\leq 10^5$ CFU/mL, live cells behaved differently than the dead ones when treated by PMA-qPCR. But, when dead cell concentration increased, a strong deviation from the real live cell concentration was noted. The critical dead/live ratio could be 10^2. This could be related to the limited capacity of PMA to completely suppress the dead cell DNA amplification, if the different parameters of this technique are not optimized before. Previous studies that have reported the influence of dead cell presence on the quantification of live *Legionella* cells - such as Qin et al. (2012), Slimani et al. (2012), and Yañez et al. (2011)- agreed with these results. When *Legionella* cells were mixed with a variable number of heat-killed cells no significant change in amplification was observed whenever the number of dead cells was lower than 10^5 CFU (Qin et al., 2012). In this case, the critical dead/live ratio seems also to be 10^2. Yañez et al. (2011) reported that the presence of dead cells affected negatively the live cell number determination in live-dead mixtures, especially when the concentration of dead cells was higher than 4 log units. In this study the critical dead/live ratio could be 10. Slimani et al. (2012) found that for a dead/live ratio higher than 10^3 PMA-qPCR overestimated the number of live *Legionella* cells. And the critical dead/live ratio could be lower for VBNC *Legionella* cells (Slimani et al., 2012). On other hand, some studies have reported that the presence of dead *Legionella* cells did not significantly affect the quantification of live cells by EMA-qPCR (Chen and Chang et al., 2010; Delgado Viscogliosi et al., 2009). Chen and Chang (2010) used a high concentration of heated cells (7 log units) in mixture samples but the dead/live ratio was not higher than 10^2. While in the Delgado Viscogliosi et al. (2009) study the concentration of heated cells in the mixture seems not to be higher than 2×10^5 CFU/mL.

It is worth of mention that the presence in high concentrations of viable cells does not seem to affect the performance of the v-qPCR technique (Chen and Chang et al., 2010; Slimani et al., 2012).

Taking into account all the aforementioned it is clear that the EMA/PMA-DNA binding efficiency is lower than what was expected. If a straightforward calculation is performed considering a cell amount of 5×10^7, a 50 μM PMA concentration, and assuming that the *Legionella pneumophila* genome size is 3.66×10^6 bp[2], there are about 82 PMA molecules available for each bp. It seems to be enough PMA molecules to bind to the DNA from damaged cells. However, Yielding et al. (1984) pointed out that in the formation of DNA-adducts the strong association alone is not sufficient to provoke an appropriate change in the properties of DNA because of complex factors such as drug distribution and metabolic disposition. For eukaryotic cells, such as lymphocytes, Cantrell and Yielding (1980) found that the distribution of EMA in the chromatin fraction was 55% in DNA, 28% in protein, and 16% in RNA. Therefore, the intercalating dyes also react with proteins, with RNA, and with cytoplasmic DNA, all of them present in prokaryotic cells. Moreover, the selectivity or preferential binding of the drugs for the mentioned cells components is not clear at all (Cantrell et al., 1979; Hixon et al., 1975). Furthermore, although experimental evidence is necessary, DNA sequence could make drug intercalation more favorable or less (Fittipaldi et al., 2012).

On other hand, little is known about ligand–DNA adduct stability. This is assumed as a not reversible binding. However, the v-PCR technique efficiency could be also be reduced by a DNA repair process (Fukunag and Yielding, 1979). Thus, many questions about how efficiently the dyes bind and the impact of binding on DNA structure and function have still not been answered and further studies will enable researcher to move toward more efficient

[2] http://www.ncbi.nlm.nih.gov/genome/genomes/416. Last access 12/07/2013.

exclusion of dead cell signal amplification. However, and as it was aforementioned, important progress has been made in the last years to find a better v-PCR protocol. Although we have not tested any conventional disinfection treatment and only a heat treatment has been applied, other authors have reported that the PMA-qPCR method may be useful to evaluate the evolution of a disinfection process by following the change of Ct (Nocker et al., 2006; Nocker et al., 2007). In this case, a high number of dead cells can be present in the sample. The limitations of the PMA-qPCR method can be overcome by the independent determination of the viable cell proportion through the measurement of fluorescence using the LIVE/DEAD® BacLightTM Bacterial Viability Kit. This method does not seem to be affected by the presence of dead cells. However, Biggerstaff et al. (2006) pointed out that auto-fluorescence and non-specific binding issues can be encountered when the method is used to test complex samples such as wastewater. Furthermore, this method is not bacterial specific. The combination of both procedures permits the quantification of live and dead cells in a mixture of known bacteria, thus becoming a useful tool to evaluate disinfection kinetics or methods in laboratory assays.

5.5. Conclusions

The herein used v-qPCR method by itself seems not to be the more suitable for the enumeration of live *Legionella pneumophila* when the concentration of dead cells is larger (in a factor approximately to 10^2), since it will lead to false estimates of live cells. Thus, the viable qPCR method, by itself without additional improvements, may not be suitable for the correct quantification of *Legionella pneumophila* in environmental samples with a high number of dead cells, and/or high contaminated samples exposed to disinfection treatments. However, some modifications should be performed in the used v-qPCR protocol - such as amplicon length, incubation time, incubation temperature, photo-activation light source, and appropriate reaction and DNA extraction buffers - in order to obtain a higher suppression of the dead cell DNA amplification.

It is also important to study real conditions in environmental samples. A dead cell concentration of 10^7 CFU/mL is rarely found in water samples. Concentration of legionellae commonly observed in environmental samples and water systems is generally lower than 10^5 CFU/mL (Declerck et al., 2007; Devos et al., 2005; Morio et al., 2008). However, high levels of live and dead *Legionella* and non-*Legionella* bacteria can be present, especially after disinfection treatments which increase the concentration of membrane injured cells. In these cases, the additional analysis of a non-concentrated sample by v-qPCR, as it is currently used in *Legionella* culture analysis from environmental samples, may be useful. Additionally, plate counts could be performed to discard false-positive results.

The inhibition of the dead cell DNA amplification signal seems to be a function of the probability of PMA entering into the cell, which could be defined by the ratio of the number of targets that were effectively reached by PMA to the total target number. It has been demonstrated that several factors affect this probability and can be optimized in order to maximize the v-qPCR signal of membrane-compromised cells in both, laboratory-grown cell samples and complex environmental samples. Factors such as dye concentration, incubation conditions, and qPCR amplicon length should be optimized, and then further studies on mixtures of known proportions of dead and live bacteria using the optimized protocols would be needed in order to verify that high levels of dead bacteria do not affect the proper discrimination.

Given that the technique is under development, further research is needed to identify and solve its limitations to improve its feasibility for routine microbial water monitoring, which implies reliable detection of few relevant microorganisms in a complex microbial background.

5.6. References

Agusti, G., Codony, F., Fittipaldi, M., Adrados, B., Morató, J. 2010. Viability determination of *Helicobacter pylori* using Propidium Monoazide quantitative PCR. Helicobacter, 15, pp. 473–476.

Agusti, G., Fittipaldi, F., Morató, J., Codony, F. 2013. Viable quantitative PCR for assessing the response of *Candida albicans* to antifungal treatment. Appl. Microbial. Biotechnol., 97, pp. 341-349.

Andorrà, I., Esteve-Zarzoso, B., Guillamón, J.M., Mas, A. 2010. Determination of viable wine yeast using DNA binding dyes and quantitative PCR. Int. J. Food Microbiol., 144, pp. 257-262.

Bae, S., Wuertz, S. 2009. Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with Propidium Monoazide. Appl. Environ. Microbiol., 75, pp. 2940-2944.

Ballard, A.L., Fry, N.K., Chan, L., Surman, S.B., Lee, J.V., Harrison, T.G., Towner, K.J. 2000. Detection of *Legionella pneumophila* using a real-time PCR hybridization assay. J. Clin. Microbiol., 38, pp. 4215-4218.

Banihashemi, A., Van Dyke, M.I., Huck, P.M. 2012. Long-amplicon propidium monoazide-PCR enumeration assay to detect viable *Campylobacter* and *Salmonella*. J. Appl. Microbiol., 113, pp. 863-873.

Behets, J., Declerck, P., Delaedt, Y., Creemers, B., Ollevier, F. 2007. Development and evaluation of a Taqman duplex real time PCR quantification method for reliable enumeration of *Legionella pneumophila* in water samples. J. Microbiol. Methods., 68, pp. 137-144.

Bej, A.K., Mahbubani, M.H., Atlas, R.M. 1991. Detection of viable *Legionella pneumophila* in water by polymerase chain reaction and gene probe methods. App. Environ. Microbiol., 57, pp. 597-600.

Biggerstaff, J.P., Puil, M.L., Weidow, B.L., Prater, J., Glass, K., Radosevich, M., White, D.C. 2006. New methodology for viability testing in environmental samples. Mol. Cell. Probes, 20, pp. 141-146.

Blooi, M., Martel, A., Vercammen, F., Pasmans, F. 2013. Combining ethidium monoazide treatment with real-time PCR selectively quantifies viable *Batrachochytrium dendrobatidis* cells. Fungal Biol., 117, pp. 156-162.

Brescia, C.C., Griffin, S.M., Ware, M.W., Varughese, E.A., Egorov, A.I., Villegas, E.N. 2009. *Cryptosporidium* propidium monoazide-PCR, a molecular biology-based technique for genotyping of viable *Cryptosporidium* oocysts. Appl. Environ. Microbiol., 75, pp. 6856-6863.

Cantrell, C.E., Yielding, K.L., Pruitt, K.M. 1979. Efficiency of photolytic binding of ethidium monoazide to nucleic acids and synthetic polynucliotides. Mol. Pharmacol., 14, pp. 322-330.

Cantrell, C.E., Yielding, K.L. 1980. Binding of ethidium monoazide to the chromatin in human lymphocytes. Biochim. Biophys. Acta, 26, pp. 173-179.

Cawthorn, D.M., Witthuhn, R.C. 2008. Selective PCR detection of viable *Enterobacter sakazakii* cells utilizing propidium monoazide or ethidium bromide monoazide. J. Appl. Microbiol., 105, pp. 1178-1185.

Chaabna, Z., Forey, F., Reyrolle, M., Jarraud, S., Atlan, D., Fontvieille, D., Gilbert, C. 2013. Molecular diversity and high virulence of *Legionella pneumophila* strains isolated from biofilms developed within a warm spring of a thermal spa. BMC Microbiol., 13:17, pp. 1-10.

Chang, B., Sugiyama, K., Taguri, T., Amemura-Maekawa, J., Kura, F., Watanabe, H. 2009. Specific detection of viable *Legionella* cells by combined use of photoactivated ethidium monoazide and PCR/real-time PCR. Appl. Environ. Microbiol., 75, pp.147-153.

Chang, B., Taguri, T., Sugiyama, K., Amemura-Maekawa, J., Kura, F., Watanabe, H. 2010. Comparison of ethidium monoazide and propidium monoazide for the selective detection of viable *Legionella* cells. Jpn. J. Infect. Dis., 63, pp. 119-123.

Chen, N.T., Chang, C.W. 2010. Rapid quantification of viable legionellae in water and biofilm using ethidium monoazide coupled with real-time quantitative PCR. J. Appl. Microbiol., 109, pp. 623-634.

Codony, F., Perez, L.M., Adrados, B., Agusti, G., Fittipaldi, M., Morató, J. 2012. Amoeba-related health risk in drinking water systems: could monitoring of amoebae be a complementary approach to current quality control strategies?. Future Microbiol., 7, pp. 25-31.

Crespo-Sempere, A., Estiarte, N., Marín, S., Sanchis, V., Ramos, A.J. 2013. Propidium monoazide combined with real-time quantitative PCR to quantify viable *Alternaria* spp. contamination in tomato products. Int. J. Food Microbiol., 165, pp. 214-220.

Declerck, P., Behets, J., van Hoef, V., Ollevier, F. 2007. Detection of *Legionella* spp. and some of their amoeba hosts in floating biofilms from anthropogenic and natural aquatic environments. Water Res., 41, pp. 3159-3167.

Delgado Viscogliosi, P., Solignac, L., Delattre, J. M. 2009. Viability PCR, a culture-independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. Appl. Environ. Microbiol., 75, pp. 3502-3512.

Devos, L., Clymans, K., Boon, N., Verstraete, W. 2005. Evaluation of nested PCR assays for the detection of *Legionella pneumophila* in a wide range of aquatic samples. J. Appl. Microbiol., 99, pp. 916-925.

Diederen, B.M.W., Jong, C.M.A.D., Marmouk, F., Kluytmans, J.A.J.W., Peeters, M.F., Van der Zee, A. 2007. Evaluation of real-time PCR for the early detection of *Legionella pneumophila* DNA in serum samples. J. Med. Microbiol. 56, pp. 94-101.

Elizaquível, P., Azizkhani, M., Sánchez, G., Aznar, R. 2013. Evaluation of *Zataria multiflora Boiss.* essential oil activity against *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes* by propidium monoazide quantitative PCR in vegetables. Food control, 34, pp. 770-776.

Fittipaldi, M., Codony, F., Morató, J. 2010a. Comparison of conventional culture and real-time quantitative PCR using SYBR Green for detection of *Legionella pneumophila* in water samples. Water SA, 36, pp. 417-423.

Fittipaldi, M., Rodriguez, N. J., Codony, F., Adrados, B., Peñuela, G., Morató, J. 2010b. Discrimination of infectious bacteriophage T4 virus by propidium monoazide real-time PCR. J. Virol. Methods, 168, pp. 228-232.

Fittipaldi, M., Pino Rodriguez, N.J., Adrados, B., Agustí, G., Peñuela, G., Morató, J. 2011. Discrimination of viable *Acanthamoeba castellani* trophozoites and cysts by propidium monoazide real-time Polymerase Chain Reaction. J. Eukaryot. Microbiol., 58, pp. 359-364.

Fittipaldi, M., Nocker, A., Codony, F. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J. Microbiol. Methods, 91, pp. 276-289.

Flekna, G., Stefanic, P., Wagner, M., Smulders, F.J., Mozina, S.S., Hein, I. 2007. Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. Res. Microbiol., 158, pp. 405–412.

Fukunag, M., Yielding, K.L. 1979. Fate during cell growth of yeast mitochondrial and nuclear DNA after photolytic attachment of the monoazide analog of ethidium. Biochem. Biophys. Research Communications, 90, pp. 582-586.

Girones, R., Ferrús, M.A., Alonso, J.L., Rodriguez-Manzano, J., Calgua, B., de Abreu Corrêa, A., Hundesa, A., Carratala, A., Bofill-Mas, S. 2010. Molecular detection of pathogens in water-the pros and cons of molecular techniques. Water Res., 44, pp. 4325-4339.

Gomez Valero, L., Rusniok, C., Buchrieser, C. 2009. *Legionella pneumophila*: population genetics, phylogeny and genomics. Infect. Genet. Evol., 9, pp. 727-739.

Graiver, D.A., Saunders, S.E., Topliff, C.L., Kelling, C.L., Bartelt-Hunt, S.L. 2010. Ethidium monoazide does not inhibit RT-PCR amplification of nonviable avian influenza RNA. J. Virol. Methods, 164, pp. 51-54.

Hayden, R.T., Uhl, J.R., Qian, X., Hopkins, M.K., Aubry, M.C., Limper, A.H., Lloyd, R.V., Cockerill, F.R. 2001. Direct detection of *Legionella* species from bronchoalveolar lavage and open lung biopsy specimens: Comparison of LightCycler PCR, in situ hybridization, direct fluorescence antigen detection, and culture. J. Clin. Microbiol., 39, pp. 2618–2626.

Hixon, S.C., White, W.E., Yielding, K.L. 1975. Selective covalent binding of an ethidium analog to mitochondrial DNA with production of petite mutants in yeast by photoaffinity labeling. J. Mol. Biology, 92, pp. 319-329.

Hussong, D., Colwell, R.R., O'Brien, M., Weiss, E., Pearson, A.D., Weiner, R.M., Burge, W.D. 1987. Viable *Legionella pneumophila* not detectable by culture on agar media. Bio/Technology, 5, pp. 947-950.

International Standard Organization. 2012. ISO/TS 12869. Water quality — Detection and quantification of *Legionella* spp. and/or *Legionella pneumophila* by concentration and genic amplification by quantitative polymerase chain reaction (qPCR). Geneva, Switzerland.

Joly, P., Falconnet, Andre, J., Weill, N., Reyrolle, M., Vandenesch, F., Maurin, M., Etienne, J., Jarraud, S. 2006. Quantitative real-time *Legionella* PCR for environmental water samples: data interpretation. Appl. Environ. Microbiol., 72, pp. 2801-2808.

Josephson, K.L., Gerba, C.P., Pepper, I.L. 1993. Polymerase chain reaction detection of non-viable bacterial pathogens. Appl. Environ. Microbiol., 59, pp. 3513–3515.

Kao, P.M., Tung, M.C., Hsu, B.C., Chiu, Y.C., She, C.Y., Shen, S.M., Huang, Y.L., Huang, W.C. 2013. Identification and quantitative detection of *Legionella* spp. in various aquatic environments by real-time PCR assay. Environ. Sci. Pollut., 20, pp. 6128-6137.

Keer, J.T., Birch, L. 2003. Molecular methods for the assessment of bacterial viability. In: Cenciarini-Borde, C., Courtois, S., La Scola, B. 2009. Nucleic acids as viability markers for bacteria detection using molecular tools. Future Microbiol., 4, pp. 45-64.

Kobayashi, H., Oethinger, M., Tuohy, M. J., Hall, G. S., Bauer, T.W. 2009. Unsuitable distinction between viable and dead *Staphylococcus aureus* and *Staphylococcus epidermis* by ethidium bromide monoazide. Lett. Appl. Microbiol., 48, pp. 633–638.

Kralik, P., Nocker, A., Pavlik, I. 2010. *Mycobacterium avium* subsp. *Paratuberculosis* viability determination using F57 quantitative PCR in combination with propidium monoazide treatment. Int. J. Food Microbiol., 141(Suppl.1), pp. S80-S86.

Levi, K., Smedley, J., Towner, K.J. 2003. Evaluation of a real-time PCR hybridization assay for rapid detection of *Legionella pneumophila* in hospital and environmental water samples. Clin. Microbiol. Infect., 9, pp. 754-758.

Martin, B., Raurich, S., Garriga, M., Aymerich, T. 2013. Effect of amplicon length in propidium monoazide quantitative PCR for the enumeration of viable cells of *Salmonella* in cooked ham. Food Anal. Methods, 6, pp. 683-690.

Mascone, C.F. 2008. *Legionella*: an invisible risk. Chemical Engineering Progress, 104, pp. 6-10.

Masters, C.I., Shallcross, J.A., Mackey, B.M. 1994. Effect of stress treatments on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain reaction. J. Appl. Bacteriol., 77, pp. 73–79.

Meng, X.C., Pang, R., Wang, C., Wang, L.Q. 2010. Rapid and direct quantitative detection of viable bifidobacteria in probiotic yogurt by combination of ethidium monoazide and real-time PCR using a molecular beacon approach. J. Dairy Res., 77, pp. 498-504.

Mérault, N., Rusniok, C., Jarraud, S., Gomez-Valero, L., Cazalet, C., Marin, M., Brachet, E., Aegerter, P., Gaillard, J.L., Etienne, J., Hermann, J.L., the DELPH-I Study Group, Lawrence, C., Buchrieser, C. 2011. Specific real-time PCR for simultaneous detection and identification of *Legionella pneumophila* serogroup 1 in water and clinical samples. Appl. Environ. Microbiol., 77, pp. 1708-1717.

Morio, F., Corvec, S., Caroff, N., Le Gallou, F., Drugeon, H., Reynaud, A. 2008. Real-time PCR assay for the detection and quantification of *Legionella pneumophila* in environmental water samples: Utility for daily practice. Int. J. Hyg. Environ. Health, 211, pp. 403-411.

Nkuipou-Kenfack, E., Engel, H., Fakih, S., Nocker, A. 2013. Improving efficiency of viability-PCR for selective detection of live cells. J. Microbiol. Methods, 93, pp. 20-24.

Nocker, A., Camper, A.K. 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl. Environ. Microbiol., 72, pp. 1997–2004.

Nocker, A., Cheung, C.Y., Camper, A.K. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Methods, 67, pp. 310-320.

Nocker, A., Sossa, K.E., Camper, A.K. 2007. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. J. Microbiol. Methods, 70, pp. 252-260.

Nogva, H.K., DrØmtorp, S.M., Nissen, H., Rudi, K. 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5`-nuclease PCR. BioTechniques, 34, pp. 804-813.

Pan, Y., Breidt, F. 2007. Enumeration of viable *Listeria monocytogenes* cells by real time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. Appl. Environ. Microbiol., 73, pp. 8028-8031.

Qin, T., Zhengan, T., Ren, H., Hu, G., Zhou, H., Lu, J., Luo, C., Liu, Z., Shao, Z. 2012. Application of EMA-qPCR as a complementary tool for the detection and monitoring of *Legionella* in different water systems. World J. Microbiol. Biotechnol., 28, pp. 1881-1890.

Rawsthorne, H., Dock, C. N., Jaykus, L. A. 2009. PCR-Based method using propidium monoazide to distinguish viable from non-viable *Bacillus subtilis* spores. Appl. Environ. Microbiol., 75, pp. 2936-2939.

Rudi, K., Moen, B., Drømtorp, S.M., Holck, L. 2005. Use of Ethidium Monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl. Environ. Microbiol., 71, pp. 1018-1024.

Sanchez, G., Elizaquivel, P., Aznar, R. 2012. Discrimination of infectious hepatitis A viruses by propidium monoazide real-time RT-PCR. Food Environ. Virol., 4, pp. 21-25.

Shi, H., Xu, W., Trinh, Q., Luo, Y., Liang, Z., Li, Y., Huang, K. 2012. Establishment of a viable cell detection system for microorganisms in wine based on ethidium monoazide and quantitative PCR. Food Control, 27, pp. 81-86.

Slimani, S., Robyns, A., Jarraud, S., Molmeret, M., Dusserre, E., Mazure, C., Facon, J.P., Lina, G., Ginevra, C. 2012. Evaluation of propidium monoazide (PMA) treatment directly on membrane filter for the enumeration of viable but non cultivable *Legionella* by qPCR. J. Microbiol. Methods, 88, pp. 319-321.

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Takade, A., Yoshida, S. 2007. Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria. Microbiol. Immunol., 51, pp. 763–775.

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki., M., Yoshida, S. 2008. Method to detect only live bacteria during PCR amplification. J. Clin. Microbiol., 46, pp. 2305-2313.

Soejima, T., Schlitt-Dittrich, F., Yoshida, S. 2011. Polymerase chain reaction length-dependent ethidium monoazide suppression power for heat-killed Enterobacteriaceae. Anal. Biochem., 418, pp. 37-43.

Vendrame, M., Iacumin, L., Manzano, M., Comi, G. 2013. Use of propidium monoazide for the enumeration of viable *Oenococcus oeni* in must and wine by quantitative PCR. Food Microbiol., 35, pp. 49-57.

Vesper, S., McKinstry, C., Hartmann, C., Neace, M., Yoder, S., Vesper A. 2008. Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). J. Microbiol. Methods, 72, pp. 180-184.

Wagner, A.O., Malin, C., Knapp, B.A., Illmer, P. 2008. Removal of free extracellular DNA from environmental samples by ethidium monoazide and propidium monoazide. Appl. Environ. Microbiol., 74, 2537–2539.

Wang, L., Li, Y., Mustapha, A. 2009. Detection of viable *Escherichia coli* O157:H7 by ethidium monoazide real-time PCR. J. Appl. Microbiol., 107, pp. 1719–1728.

Wellinghausen, N., Frost, C., Marre, R. 2001. Detection of *Legionellae* in hospital water samples by quantitative real-time LightCycler PCR. Appl. Environ. Microbiol., 67, pp. 3985–3993.

Wilson, D.A., Yen-Lieberman, B., Reischl, U., Gordon, S.M., Procop, G. 2003. Detection of *Legionella pneumophila* by real-time PCR for the *mip* gene. J. Clin. Microbiol., 41, pp. 3327-3330.

Yañez, M.A., Nocker, A., Soria-Soria, E., Múrtula, R., Martinez, L., Catalán, V. 2011. Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. J. Microbiol. Methods, 85, pp. 124-130.

Yaradou, D.F., Hallier-Soulier, S., Moreau, S., Poty, F., Hillion, Y., Reyrolle, M., André, J., Festoc, G., Delabre, K., Vandenesch, F., Etienne, J., Jarraud, S. 2007. Integrated real-time PCR for detection and monitoring of *Legionella pneumophila* in water systems. Appl. Environ. Microbiol., 73, pp. 1452–1456.

Yasunaga, A., Yoshida, A., Morikawa, K., Maki, K., Nakamura, S., Soh, I., Awano, S., Ansai, T. 2013. Monitoring the prevalence of viable and dead cariogenic bacteria in oral specimens and in vitro biofilms by qPCR combined with propidium monoazide. BMC Microbiology, 13:157, pp. 1-9.

Yielding, L.W., Yielding, K.L., Donoghue, J.E. 1984. Ethidium binding to deoxyribonucleic acid: spectrophotometric analysis of analogs with amino, azido, and hydrogen substituents. Biopolymers, 23, pp. 83-110.

Yu, V., Plouffe, J.F., Pastoris, M.C., Stout, J.E., Schousboe, M., Widmer, A., Summersgill, J., File, T., Heath, C.M., Paterson, D.L., Chereshsky, A. 2002. Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionelosis: an international collaborative survey. J. Infect. Dis., 186, pp. 127-128.

Chapter 6

An approach to provide an improved interpretation of viable cells level estimation by qPCR*

Selective nucleic acid intercalating dyes -ethidium monoazide (EMA) and propidium monoazide (PMA)- represent one of the most successful recent approaches to detect viable cells (as defined by an intact cell membrane) by qPCR, and have been effectively evaluated in different microorganisms. However, some practical limitations were found, especially in environmental samples. The aim of this chapter is to propose a strategy for overcoming some of these problems. An approach centered on the combination of three qPCR amplifications for each sample that should provide an improved estimation of the number of viable cells is presented. This approach could be useful especially when it is difficult to determine *a priori* how to optimize methods using PMA or EMA. Although further studies are required to improve v-qPCR methods, the concept as outlined here presents an interesting future research direction.

*An early version of this chapter was published as:

Fittipaldi, M., Codony, F., Adrados, B., Camper, A.K., Morato, J. 2011. Viable real-time PCR in environmental samples: can all data be interpreted directly? Microb. Ecol., 61, pp. 7-12.

6.1. Introduction

Polymerase chain reaction (PCR) is a rapid and sensitive technique for microbial detection. However, until a few years ago, it was assumed that a major disadvantage of this method was its inability to differentiate between live and dead cells (Wang and Levin, 2006) and the overestimation of potentially viable biomass. After the first publication of a real time PCR (qPCR) procedure using ethidium monoazide (EMA) this premise began to change (Nogva et al., 2003).

The utilization of selective nucleic acid intercalating dyes, like EMA and propidium monoazide (PMA), has been suggested as a means to reduce PCR signals from DNA originated from dead cells (Cawthorn and Witthuhn, 2008; Nocker and Camper, 2006; Nogva et al., 2003; Rudi et al., 2005). Therefore, it is one of the most successful approaches to detect live cells by PCR or qPCR (herein called v-qPCR). The approach is based on membrane integrity to distinguish between live and dead cells. Theoretically, selective nucleic acid intercalating dyes should only penetrate into membrane-compromised cells or dead cells. The presence of an azide group is believed to permit crosslinking of the dye to the DNA after exposure to strong visible light. The photolysis of EMA and PMA converts the azide group into a highly reactive nitrene radical, which can react with any organic molecule in its proximity including the bound DNA. In this bound state, the DNA cannot be amplified by PCR (Nocker and Camper, 2009; Rudi et al., 2005). At the same time when the crosslinking with DNA occurs, the light reacts unbound excess dye with water molecules. The resulting hydroxylamine is no longer reactive, so the DNA from cells with intact membranes is supposedly not modified during the DNA extraction procedure (Nocker et al., 2009).

The use of EMA or PMA has been effectively evaluated in different bacteria (Agustí et al., 2013; Agustí et al., 2010; Bae and Wuertz, 2009; Cawthorn and Witthuhn, 2008; Delgado Viscogliosi et al., 2009; Desfossés-Foucault et al., 2012; Dinu and Bach, 2013; Kaushik and Balasubramanian, 2013; Nam et al., 2011; Pan and Breidt, 2007; Soejima et al., 2012; Soejima et al., 2007; Yang et al., 2012), spores (Rawsthorne et al., 2009), fungi (Vesper et al., 2008), yeast (Andorrà et al., 2010; Shi et al., 2012; Willenburg and Divol, 2012), protozoa (Brescia et al., 2009; Fittipaldi et al., 2011), and viruses (Fittipaldi et al., 2010; Graiver et al., 2010; Kim and Go, 2012; Parshionikar et al., 2010; Sanchez et al., 2012). However, there is evidence demonstrating that v-qPCR using DNA-intercalating dyes has practical and theoretical limitations especially when applied to environmental samples (Nocker et al., 2007a; Pisz et al., 2007; Varma et al., 2009; Wagner et al., 2008). In some cases most drawbacks may be reduced considerably by the development of precise procedures adapted to each sample or microorganism, and researchers are considering the use of this approach in their present and future work. It is clear that with the use of these techniques our vision of microbial dynamics in most areas of microbiology, including environmental and clinical microbiology and quality control, will be more exact or at least quite different.

Despite this exciting perspective, to improve the application of these dyes for complex environmental use, a critical discussion about the need to establish common interpretation of v-qPCR in environmental samples is warranted. The ideal situation (Figure 6.1 (1)) is when all DNA present in the sample may be detected by qPCR and all DNA from live microorganisms may be detected by v-qPCR. As expected, several critical points need to be considered (Figure 6.1 (2)). Firstly, the dye is not supposed to penetrate live cells. However, some studies have demonstrated that EMA may penetrate cells with intact membranes (Cawthorn and Witthuhn, 2008; Chen and Chang, 2010; Flekna et al., 2007; Kobayashi et al., 2009; Nocker and Camper, 2006; Nocker et al., 2006), with the extent of EMA uptake by intact cells dependent on the bacterial species (Flekna et al., 2007; Nocker et al., 2006) and the EMA concentration (Meng et al., 2010; Wang et al., 2009).

PMA has been proposed as a more appropriate alternative (than EMA) due to a comparative study showing that PMA is efficiently excluded from cells with intact cell membranes (Nocker et al., 2006). For that reason, PMA was used in this work. It is also probable that DNA-intercalating dyes will have access to the DNA in live cells with reversibly damaged

membranes; these cells are likely to be present in environmental samples. In both of these cases cross-linkage will produce false negative results. This is fraction *a* in (2) and (3) in Figure 6.1. Secondly, as it was discussed in Chapter 5 of this dissertation, dye or light may not be able to penetrate all dead microorganisms producing false-positive results and overestimating the number of live cells (Fittipaldi et al., 2011; LØvdal et al., 2011; Wagner et al., 2008). This false-positive results are represented by fraction *b* in (2) and (3) in Figure 1.6. An example of a condition leading to false-positives would be the presence of high levels of suspended solids or biomass in water samples that could inhibit the cross-linking step by light activation since the radiation probably will not be able to penetrate through the liquid (Varma et al., 2009). Likewise, for central cells within clusters, biofilms (Pisz et al., 2007), cells embedded in encrustations or precipitates, nucleic DNA from eukaryotes, cysts or other resistant forms, and cells inside protozoa (symbiotic or parasites), the penetration of dye into cells might be limited or not occur. Optimization of the v-qPCR method using an increased amplicon length (Banihashemi et al., 2012; Luo et al., 2010; Schnetzinger et al., 2013), a higher dye concentration (Bae and Wuertz, 2009; Fittipaldi et al., 2011; Nocker et al., 2006), longer incubation times (Fittipaldi et al., 2011; Nkuipou-Kenfack et al., 2013; Rawsthorne et al., 2009; Vesper et al., 2008), and, only for PMA, higher incubation temperature (Nkuipou-Kenfack et al., 2013) has shown to be useful reducing false-positives. Furthermore, the dye could undergo chemical adsorption onto different compounds present in the sample. Another specific example is that EMA shows a variable yield of the photo-crosslinking reaction at different salt concentrations (Bolton and Kearns, 1978). Subsequently, it is likely that halophiles will need customized procedures to maximize dye performance and minimize cell death during treatment as a consequence of osmotic alterations.

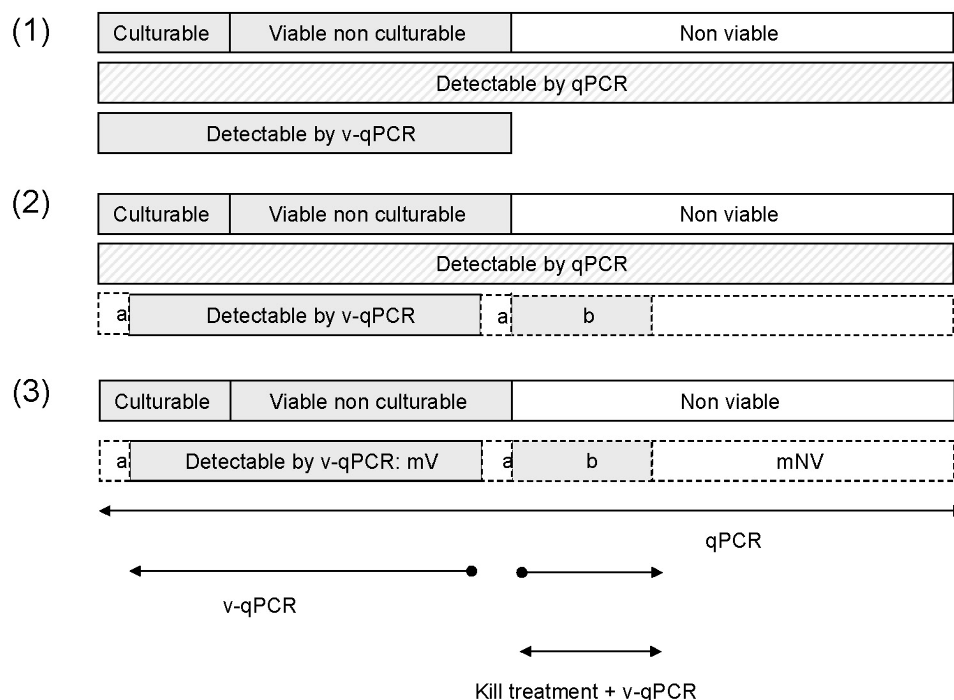


Figure 6.1. Different theoretical possibilities when photoactivable intercalating dyes are used for v-qPCR. (1) Theoretical approach: v-qPCR detects all live cells. (2) Viable qPCR method has limitations. Fraction *a*: the dye may be able to penetrate into live or reversibly damaged cells; fraction *b* not all DNA from dead cells is inactivated by pretreatment with the dye. (3) Approach for the estimation of the minimum number of viable cells (mV) using a combination of three qPCR assays for each sample. mNV: minimum number of non-viable cells.

As shown in (3) in Figure 6.1, the current v-qPCR method will detect the maximum number of live cells, which includes the minimum number of viable cells (mV) and false-positive live cells (fraction *b*). Traditional qPCR detects all organisms, including live and dead cells. The difference between the two methods gives the maximum number of dead cells which includes the minimum number of nonviable cells (mNV) and false-negative dead cells (fraction *a*). An improvement of the detection of the live cell fraction can be attained by using an additional qPCR approach (Figure 6.1 (3)). First, a rough estimation of fraction *a* for a specific organism can be obtained using qPCR, v-qPCR, and pure cultures in exponential growth, and assuming that this situation represents the optimal physiological condition for intact membranes. The qPCR results would give the minimum number of true negative cells, and the difference between qPCR and v-qPCR results would give an estimation of the fraction of false negatives (fraction *a*), that should be negligible. The values for fraction *b* can be estimated with an additional assay that consists of killing all cells and combining with a subsequent v-qPCR test. The used killing method should affect membrane integrity. The value of "kill treatment + v-qPCR" (herein called k&v-qPCR), assuming that all cells are killed, would give an estimation of the fraction of false positives (fraction *b*). Considering the estimate of fraction *a* and fraction *b*, the minimum live cell number can be calculated. This number will be a more accurate representation of the number of organisms in a sample that may pose a public health concern or act as an indicator of the quality of a product or the efficacy of a disinfection technology. By also using a qPCR assay without PMA, the difference between qPCR and v-qPCR along with the estimation of fraction *a* will let us more closely estimate the minimum number of dead cells.

The objective of this work was to show that there exist many factors that should be considered in the application of v-qPCR, and to stimulate research and discussion regarding these issues. We present preliminary data that outline the approach explained above that it is based on the combination of three qPCR amplifications for each sample, which can improve viable cell number estimation using nucleic acid amplification methods. At a minimum, these results will lead to a better understanding and a more realistic interpretation of the number of live cells in a sample.

6.2. Materials and Methods

6.2.1. Experimental assays

The three-pronged qPCR approach was used to determine the viability of *Legionella pneumophila*, *Bacteroides* spp., and *Escherichia coli* in artificially inoculated treated secondary wastewater effluent after disinfection.

Legionella pneumophila serogroup 1 (NCTC12821) was cultured in GVPC agar (Oxoid, Hampshire, UK). Once the culture was in exponential growth phase (3-day culture), a bacterial suspension was prepared by transferring single colonies into a tube with sterile saline solution and adjusting the optical density (OD_{600}) to 0.2, which approximately corresponds to a concentration of 10^8 CFU/mL, as confirmed by plate count. A decimal dilution of the bacterial suspension was inoculated in 250 L of a secondary wastewater effluent to obtain an approximate concentration of 10^2 CFU/mL. The treated secondary wastewater was held in a 20 m^3 reservoir tank that fed two different disinfection systems; chlorination and an advanced oxidation technology (AOT), photocatalysis, which utilizes titanium dioxide, photolysis, and photocatalytic decomposition by ultraviolet light. Each disinfection system consisted of a 250 L tank, where the *Legionella pneumophila* inoculation took place, a recirculation loop and a disinfection step – a chlorinator for chlorination and a Benrad water purification facility (Gadelius, Stockholm, Sweden) for the AOT system. The Benrad facility has a simple structure, consisting of a titanium pipe with a titanium oxide layer on the inner wall and an ultraviolet lamp placed in the center. Both disinfection systems work automatically by means of a programmable controller. Different treatments can be applied by changing chlorine concentration and recirculation times for chlorination or AOT.

For these experiments, three disinfection treatments were evaluated: 3 mg/L of chlorine and 30 minutes of recirculation, 3 mg/L of chlorine and 60 min of recirculation and AOT with a recirculation time of 60 minutes. Water samples of 1 L were collected in sterilized bottles with sodium thiosulfate (30 mg/L).

Bacteroides spp. and *Escherichia coli* determination using the triple approach was performed in a second experiment. In that case, the 250 L tanks filled with secondary wastewater effluent were inoculated with 0.5 L of non-disinfected wastewater. Two disinfection treatments were evaluated: 1 mg/L of chlorine and 60 minutes of recirculation and AOT with a recirculation time of 60 minutes. Samples were removed at different times (0, 15, 30, 45, and 60 min). Water samples of 1 L were collected in sterilized bottles with sodium thiosulfate (30 mg/L).

6.2.2. Sample processing

For *Legionella* determination, each sample of 300 mL was concentrated by membrane filtration using a nylon membrane (0.45 µm pore diameter, Merck Millipore, Darmstadt, Germany). Cells were resuspended in 7 mL of saline solution by vigorous vortexing for 60 seconds with 5 glass beads (5 mm diameter) and sonication for 3 minutes in an ultrasound water bath – 40 W power, 40 kHz ultrasound frequency- (JP Selecta, Barcelona, Spain). The cell suspension was split in three aliquots of 2 mL each. They were concentrated by centrifugation (14,500 rpm for 5 min) using a minicentrifuge (Minispin Plus-Eppendorf, Hamburg, Germany) and discarding the supernatant to obtain a pellet.

For *Bacteroides* spp. and *Escherichia coli* determination, each sample of 400 or 500 mL was concentrated by membrane filtration using a nylon membrane (0.45 µm pore diameter, Millipore). Cells were re-suspended in 10 mL of saline solution by vigorous vortexing for 60 seconds with 5 glass beads (5 mm diameter) and sonication for 3 minutes in an ultrasound water bath - 40W power, 40 kHz ultrasound frequency- (JP Selecta, Barcelona, Spain). The cell suspension was split in three aliquots of 3 mL each. They were concentrated by centrifugation (14,500 rpm for 5 min) using a minicentrifuge (Minispin Plus-Eppendorf, Hamburg, Germany) and discarding the supernatant to obtain a pellet.

6.2.3. PMA treatment

One of the aliquots was treated with PMA for the v-qPCR assay. Briefly, PMA (Biotium, Inc., Hayward, California, US) was dissolved in 20% dimethyl sulfoxide (DMSO, Sigma, Madrid, Spain) to create a stock concentration of 2 mM and stored at -20ºC in the dark. The bacterial pellet was resuspended with 190 µL of 1X phosphate-buffered saline, pH 7.4 (PBS) in a propylene 1.5-mL minicentrifuge tube and 10 µL of 2 mM PMA stock solution was rapidly added in a darkened room. In the case of *Bacteroides* spp. and *Escherichia coli* determination, 195 µL of PBS and 5 µL of PMA were used. The resultant cell suspension was incubated in agitation (350 rpm, Thermomix, Eppendorf, Hamburg, Germany) for 5 min in the dark at 25 ºC to allow PMA to enter into the cells with compromised or damaged membranes. The samples were then photoactivated for 15 min using PhAST blue system (GenIUL, Barcelona, Spain). After photo-induction of cross-linking, cells were pelleted by centrifugation at 14,500 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 200 µL of PBS. The second aliquot was exposed to a killing method that affects membrane integrity. In the case of *Legionella* determination, exposure to isopropanol (final concentration, 70%) for 15 minutes was used. Isopropanol treatment induces membrane damage; the assumption that isopropanol kills the cells not inactivated by disinfection was made, since this is the same approach used in the development of the v-qPCR method (Nocker et al., 2006). Isopropanol was removed by harvesting cells by centrifugation at 14,500 rpm for 5 min *prior* to resuspension in 200 µL of PBS. After that, the bacterial suspension was treated with PMA following the protocol described above and was used for the k&v-qPCR assay. Loss of culturability of isopropanol treated cells was verified by streaking 200 µL of cell suspension and dilutions on GVPC agar plates (Oxoid, Hampshire, UK) followed by incubation at 37 ºC for 10 days. In the case of *Escherichia coli* and

Bacteroides determination, the aliquots were exposed to 90 ºC during 10 min. Loss of culturability of heat treated *Escherichia coli* cells was verified by streaking 200 µL of cell suspension and dilutions on Chromocult Agar plates (Merck, Darmstadt, Germany) followed by incubation at 37 ºC for 24 h. The third aliquot also was re-suspended in 200 µL PBS and was used for the qPCR assay.

6.2.4. DNA extraction and quantitative PCR quantification

In all cases, DNA was extracted with EZNA tissue DNA purification kit (Omega Bio-Tek, Norcross, US) according to the manufacturer's instructions (E.Z.N.A.® Tissue DNA Kit Handbook, 2012).

Quantitative PCR analysis was performed on a Ligthcycler 1.5 (Roche Molecular Diagnostic, Manheim, Germany) in a 20 µL reaction volume.

The reaction mixture to detect and quantify *Legionella pneumophila* was composed of 10 µL of FastStart Taqman Probe Master (Roche Molecular Diagnostic, Manheim, Germany), 0.4 U of Uracil-DNA-glycosylase (New England BioLabs, Ipswich, USA), 9 µL of genomic DNA extract, 0.45 µM of each primer (Table 6.1), and 0.1 µM of *mip*-specific Taqman hybridization probe (Table 6.1) labeled at the 5′ end with a FAM reporter dye and at the 3′ end, with a non-fluorescent quencher and conjugated to a MGB (Behets et al., 2007). The experimental protocol consisted of one step of 2 min at 50 ºC to allow UDG to break down the possible contaminating amplicons, one step of 15 min at 95 ºC for Taq polymerase activation, and 45 cycles (95 ºC for 15 s, 60 ºC for 60 s) for DNA amplification. In this case qPCR using a Taqman probe was used instead of SYBR Green to avoid any possible false positive amplification due to primer-dimer formation, so facilitating the result analysis.

The DNA used as standard reference for *Legionella* quantification was prepared according to AFNOR XP T90-471 (AFNOR, 2006). *Legionella pneumophila* serogroup 1 (NCTC12821) was used as a reference strain. A standard DNA curve was established using a 3-day culture in GVPC agar (Oxoid, Hampshire, UK). Once the culture was ready, a bacterial suspension was prepared by transferring single colonies into a tube with sterile saline solution and adjusting the OD_{600} to 0.2, which approximately corresponds to a concentration of 10^8 CFU/mL, as confirmed by plate count. Serial ten-fold dilutions were prepared from the bacterial suspension using sterile saline solution to obtain the set of dilutions that was later used for the standard curve. DNA also was obtained with the EZNA tissue DNA purification kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions (E.Z.N.A.® Tissue DNA Kit Handbook, 2012). Amplification efficiency (100%) was estimated by means of the slope calculation method from a calibration dilution curve (Rasmussen, 2001).

Determination of *Bacteroides* spp. was performed by using the primers and probes (Table 6.1) that have previously been described and validated (Layton et al., 2006). Nine µL of DNA were mixed with 0.4 µM of each primer, 0.2 µM FAM labeled probe concentration, and 10 µL of FastStart Taqman Probe Master (Roche Molecular Diagnostics, Mannheim, Germany). The amplification conditions were: 1 step of 95 ºC for 10 minutes, and then 45 cycles of 95 ºC for 15 s followed by 60 ºC for 1 minute.

Bacteroides fragilis (ATCC 51477) were cultured overnight in Brain Heart Infusion Agar (BHIA, Merk, Darmstadt, Germany) under anaerobic conditions in anaerobic jar (Merck, Darmstadt, Germany). Once the culture was ready, a bacterial suspension was prepared by transferring single colonies into a tube with sterile water. The turbidity was optically measured with a spectrophotometer and adjusted to 0.2 absorbance units at 600 nm, corresponding to approximately 10^8 CFU/mL. Cell concentration was also determined by plating on BHIA agar. The amplification efficiency was 99%.

The primers used for *Escherichia coli* determination are listed in Table 6.1. The reaction mixture consisted of 10 µL of DNA, 0.4 µM of each primer, 0.2 U of uracil-DNA-glycosylase (UDG, New England BioLabs, Suffolk, UK), and 10 µL of FastStart SYBR Green Master (Roche Molecular Diagnostics, Mannheim, Germany). The amplification procedure included an acivation step of 95 ºC for 10 minutes, 45 cycles of amplification (95 ºC for 15 s, 60 ºC for

60 s, and 72 ºC for 30 s), and a melting temperature ramp from 65 to 95 ºC at 0.1 ºC per second.

Escherichia coli (NCTC 10537) were cultured in Chromocult Agar (Merk, Darmstadt, Germany) at 37 ºC for 24 h. Once the culture was ready, a bacterial suspension was prepared by transferring single colonies into a tube with sterile water. The turbidity was optically measured with a spectrophotometer and adjusted to 0.2 absorbance units at 600 nm, corresponding to approximately 10^8 CFU/mL. Cell concentration was also determined by plating on BHIA agar. The amplification efficiency was 91%.

For each assay, the threshold cycle (Ct) was determined to quantify each DNA product. Quantification was performed including one or two external standards in each set of qPCR experiments. The cell number of each sample was determined by comparison to each standard. Each sample was tested in duplicate and mean values were calculated. A negative control, PCR-grade water (Qiagen, Hilden, Germany), was included in all assays.

Statistical analyses to calculate mean values and the standard error were performed using Microsoft Excel.

Table 6.1. Oligonucleotide primers and probes used in to detect *Legionella pneumophila mip* gene, *Bacteriodes* spp. 16S rRNA gene, and *Escherichia coli uidA* gene.

Strain	**Oligonucleotide name**	**Sequence (5'-3')**	**Size (bp)**	**Reference**
Legionella pneumophila	*mip*-LPQF	TTCATTTGYTGYTCGGTTAAAGC	66	Behets et al., 2007
	mip-LPQR	AWTGGCTAAAGGCATGCAAGAC		
	mip-LPQP	AGCGCCACTCATAG		
Bacteroides spp.	AllBac296F	GAGAGGAAGGTCCCCCAC	106	Layton et al., 2006
	AllBac412R	CGCTACTTGGCTGGTTCAG		
	AllBac375Bhqr	CCATTGACCAATATTCCTCACTGC TGCCT		
Escherichia coli	UAL1939b	ATGGAATTTCGCCGATTTTGC	166	Heijnen and Medema, 2006.
	UAL2105b	ATTGTTTGCCTCCCTGCTTGC		

6.3. Results

The herein modified v-qPCR approach was used to detect *Legionella pneumophila*, *Bacteroides* spp., and *Escherichia coli* suspended in a complex environmental water (treated secondary wastewater effluent) disinfected with chlorine and an advanced oxidation protocol. Wastewater without disinfection treatment was used as a control.

Legionella pneumophila, *Bacteroides* spp, and *Escherichia coli* were detected by qPCR. And for the last, plate count was also performed. Samples included (i) qPCR without PMA pre-treatment to give an estimate of the total target bacteria population in wastewater samples with and without disinfection; (ii) qPCR with PMA pre-treatment to give an estimate of the viable population in the disinfected samples and wastewater without disinfection sample; and (iii) qPCR with PMA pre-treatment after isopropanol or heat treatment of the wastewater samples with and without disinfection to inactive the remaining organisms and to obtain an estimate of the minimum viable cell number.

6.3.1. *Legionella pneumophila* determination

Regarding the variability among qPCR estimates of "the total population", we found that the coefficient of variation was 0.95%. The results showed that for the type of analyzed samples and for our target organism, a fraction of viable cells may be due to false-positive amplification (Figure 6.2). Moreover, we observed that the fraction of false-positives increased with the number of dead cells present in the sample. For example, after 30 min of treatment with chlorine, about 1.9% of the total number of *Legionella pneumophila* cells were viable and about 40% of these live cells may give a false-positive result if only a direct v-qPCR was performed. However, after 60 min of treatment with photocatalysis, about 12.5% of cells were viable and about 7.5% of live cells may be false-positives if only a direct v-qPCR was performed. Consequently, the data obtained using the additional step of isopropanol treatment were encouraging and indicated that the approach presented in this work is reasonable.

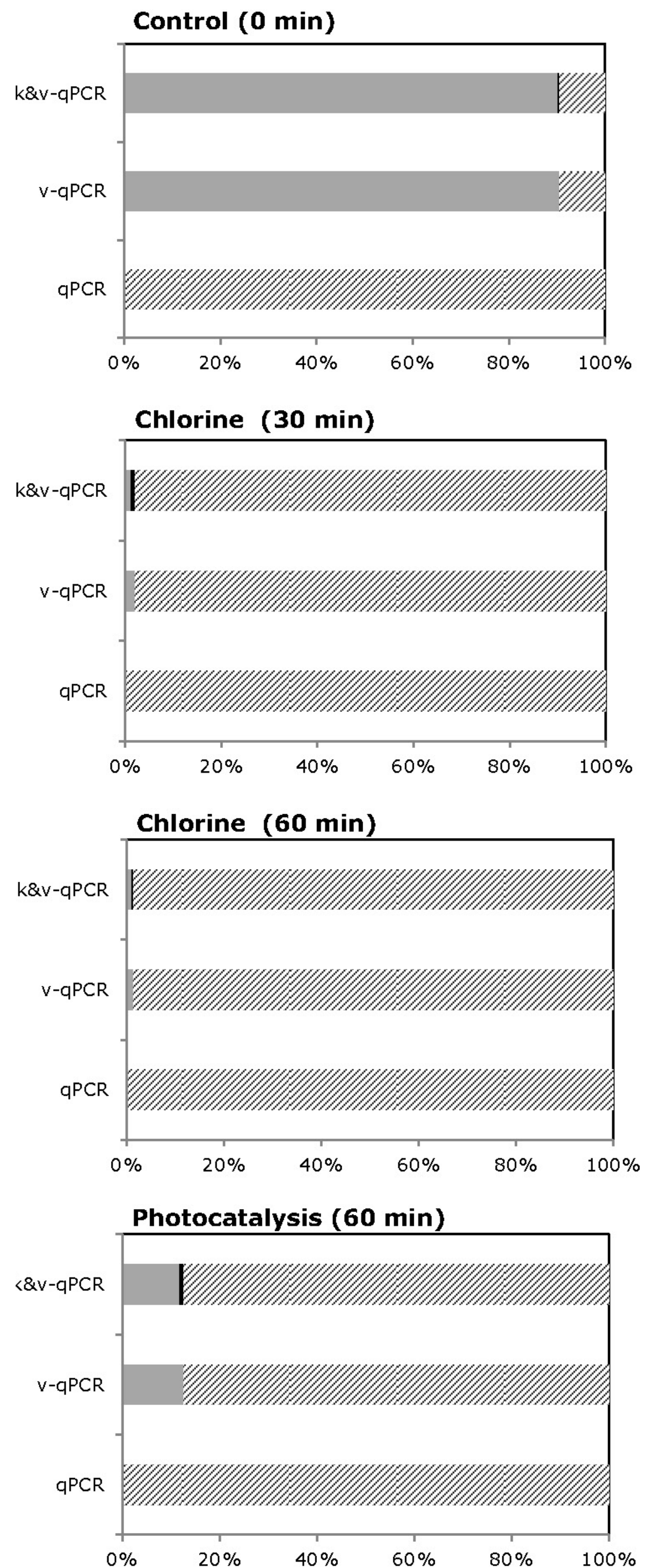


Figure 6.2. Presence of *Legionella pneumophila* in reclaimed water samples measured using three qPCR assays: rqPCR, PMA treatment and qPCR (v-qPCR), killing isopropanol treatment and v-qPCR (k&v-qPCR). Gray bars show the result of *Legionella* viable level after v-qPCR assay, and the minimum viable fraction present in the analyzed sample in the case of k&v-qPCR assay. Black bars show the false positive *Legionella* levels after k&v-qPCR. Hatched gray bars represent total *Legionella* number cells for the qPCR assay and the dead cell number for both, v-qPCR and k&v-qPCR assays.

6.3.2. *Bacteroides spp.* determination

Chlorination (1 mg/L) and photocatalysis were used to treat secondary wastewater effluent mixed with raw wastewater (0.5 L/250 L). *Bacteroides* spp. were detected and quantified in these samples. A diminution in the number of cells detected by qPCR was observed in chlorine treated samples (Figure 6.3). It can be due to DNA damage and loss induced by chlorination. It also was observed, but in a lesser extent, for the photocatalysis treatment results.

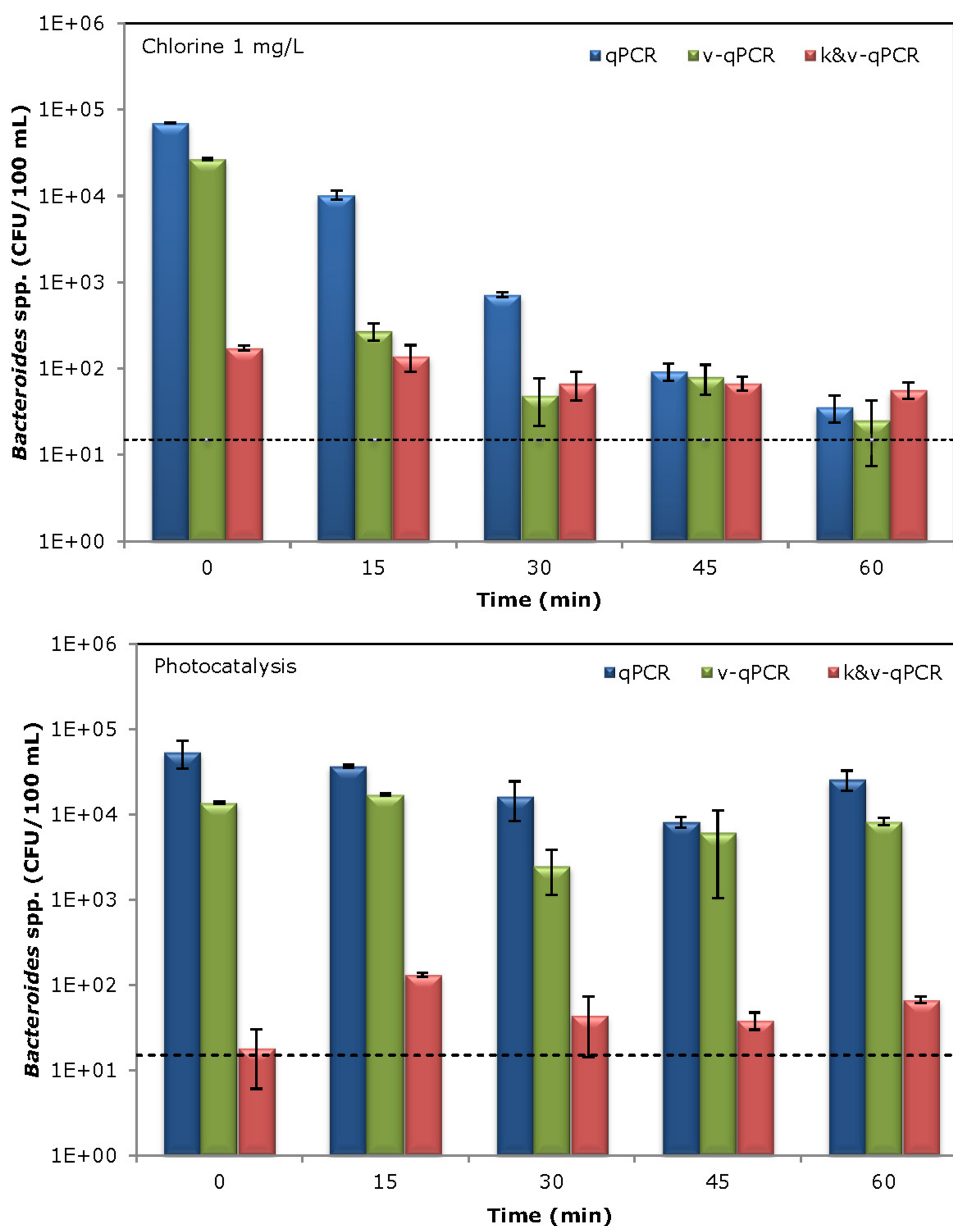


Figure 6.3. Determination of *Bacteroides* spp. reclaimed water samples measured using three qPCR assays: qPCR, PMA treatment and qPCR (v-qPCR), and killing heat treatment and v-qPCR (k&v-qPCR). Each bar represents the cell number mean value. The error bars correspond to the standard deviation of two replicate samples. The dashed line is the qPCR quantification limit, 50 CFU/100 mL.

The complete amplification signal reduction from heat treated cell was not observed when the k&v-qPCR approach was applied. It could be an indication of presence of false-positive results in the fraction of viable cells detected by v-qPCR. In the control sample, the fraction of possible false-positive results was low. About 38% of the total numbers of *Bacteroides* cells (considering qPCR result as the total cell number) were viable and about 0.25% were false-positive results. That means that about 0.65% of these live cells may be false-positive if only a direct v-qPCR was performed (Figure 6.4). Similar results were found when photocatalysis treatment was performed (Figure 6.5). However, when chlorinated samples were analyzed the presence of false-positive results in the live cell fraction seemed to be more significant. In the samples treated with 1 mg/L chlorine for 15 min, about 50% of the live cells might be false positive when only v-qPCR is used, and this fraction could be 100% for samples treated with chlorine during 30 min (Figure 6.4).

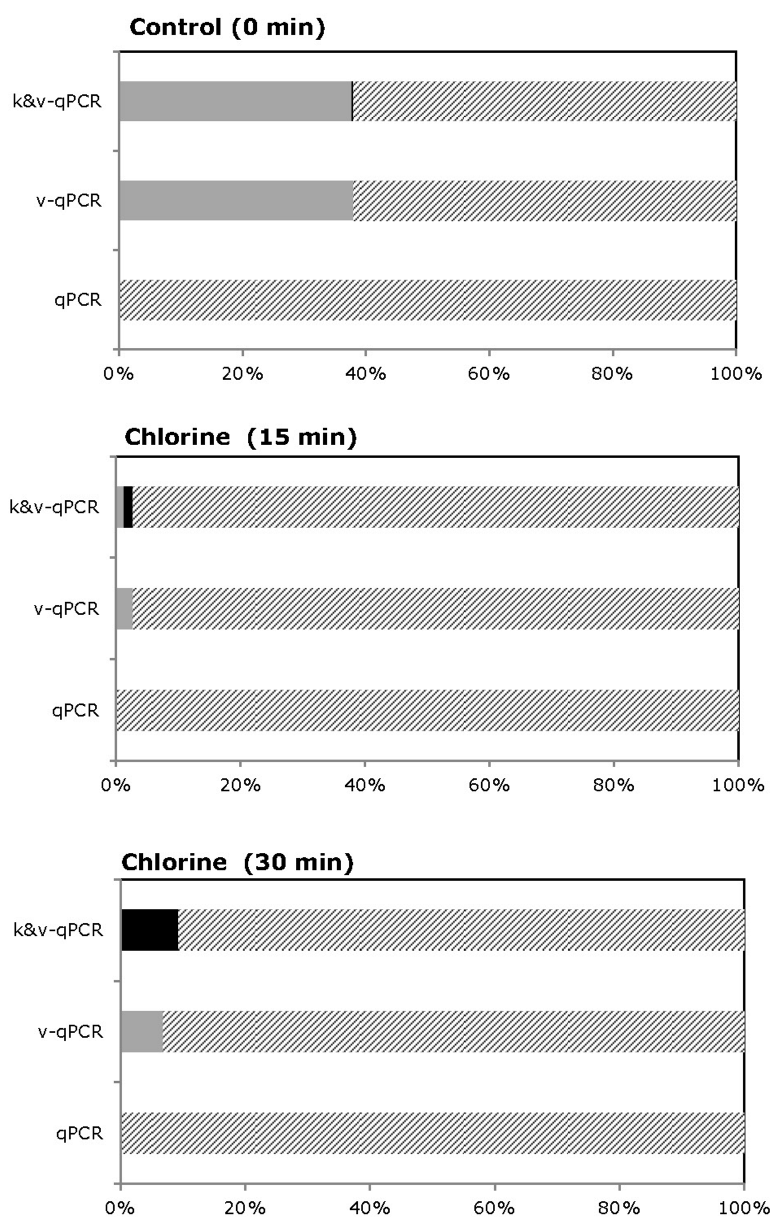


Figure 6.4. Presence of *Bacteroides* spp. in reclaimed water with chlorine disinfection *in situ* samples. Cell number was measured using three qPCR assays: real-time PCR (qPCR); PMA treatment and qPCR (v-qPCR); and killing heat treatment and v-qPCR (k&v-qPCR). Gray bars show the result of *Bacteroides* viable levels after each assay. Black bars show the possible false-positive *Bacteroides* levels after k&v-qPCR. Hatched gray bars represent total *Bacteroides* cells number for the qPCR assay, and the dead cell number for both, v-qPCR and k&v-qPCR assays. Results were obtained from replicate samples.

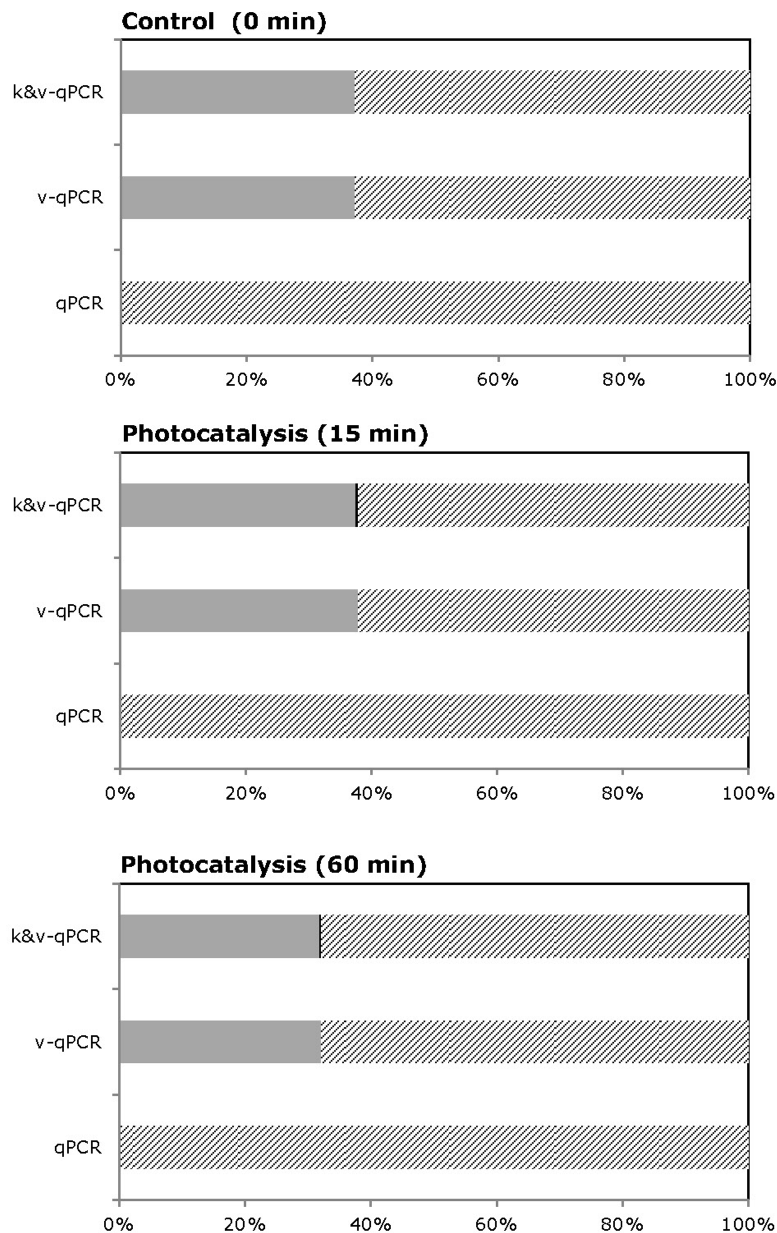


Figure 6.5. Presence of *Bacteroides* spp. in reclaimed water with photocatalysis disinfection in situ samples measured using three qPCR assays: real-time PCR (qPCR), PMA treatment and qPCR (v-qPCR), killing heat treatment and v-qPCR (k&v-qPCR). Gray bars show the result of *Bacteroides* viable levels after each assay. Black bars show the possible false positive *Bacteroides* levels after k&v-qPCR. Hatched gray bars represent total *Bacteroides* cells number for the qPCR assay, and the dead cell number for both, v-qPCR and k&v-qPCR assays. Results were obtained from replicate samples.

6.3.3. *Escherichia coli* determination

The triple approach proposed in this study and culture technique were performed to determine the *Escherichia coli* in disinfected wastewater samples. Similarly to *Bacteroides* spp. results, chlorine treatment reduced the live cell number and caused DNA loss (Figure 6.6).

In this case, culture results provided more information. In the control sample, a false-negative fraction might be present in the dead cell number determined by v-qPCR and k&v-qPCR considering the number of culturable cells. About 15% and 22% of the total *Escherichia coli* cell number (considering qPCR result as the total cell number) were false-negative results, for v-qPCR and k&v-qPCR respectively. That means that about 0.35 log units of live cells were underestimated, if only these direct viable qPCR techniques are performed (Figure 6.7).

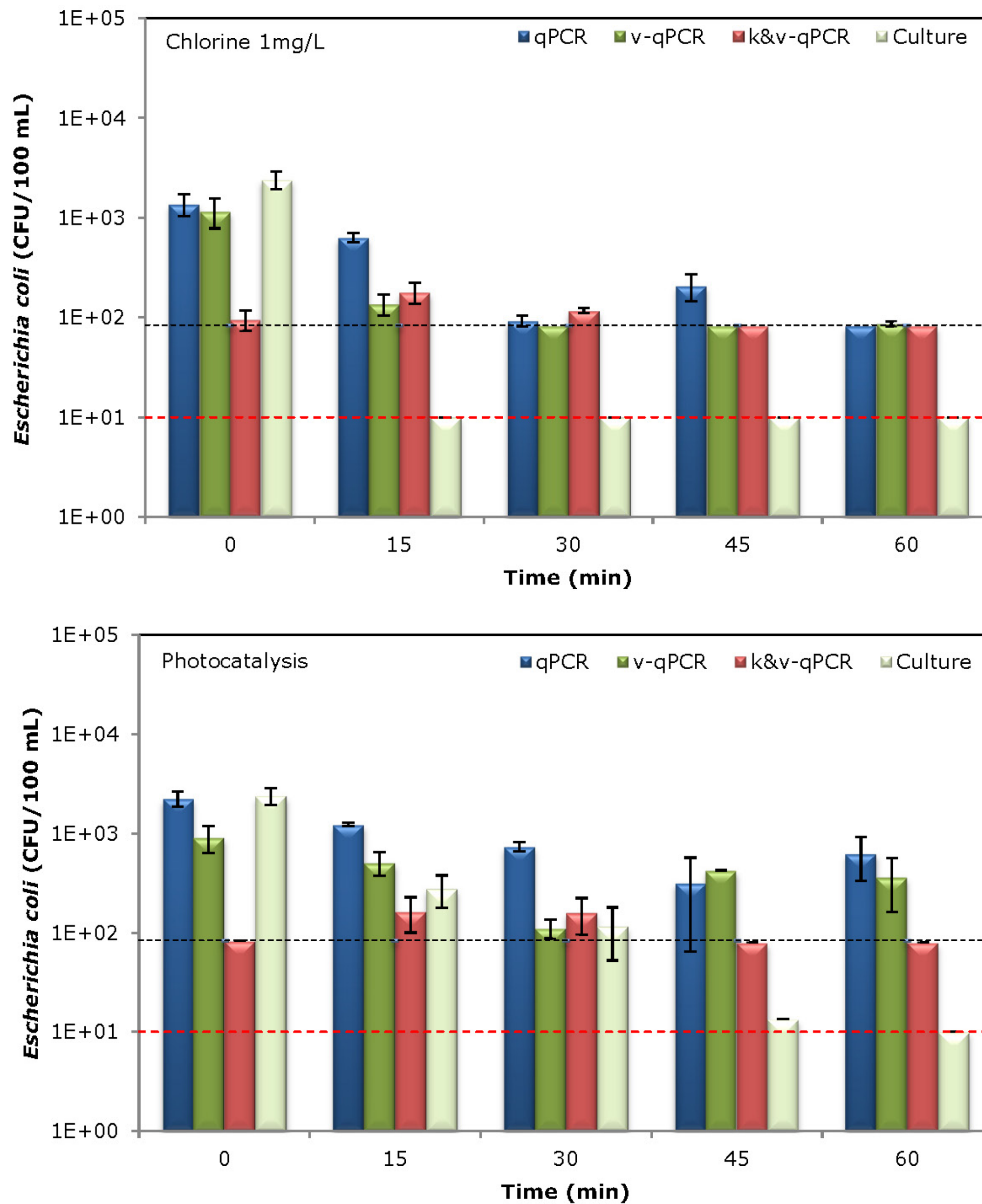


Figure 6.6. Determination of *Eschericha coli* in reclaimed water samples measured using three qPCR assays: qPCR, PMA treatment and qPCR (v-qPCR), and killing heat treatment and v-qPCR (k&v-qPCR). Each bar represents the cell number mean value. The error bars correspond to the standard deviation of two replicate samples. The black dashed line is the quantification and detection limit of qPCR technique, 84 CFU/100 mL. The red dashed line is the detection limit of culture technique, 10 CFU/100 mL.

When chlorinated samples were analyzed, the presence of false-positive results in the live cell fraction seems to be significant. In the samples treated with 1 mg/L chlorine for 15 min, about 100% of the detected live cells might be false-positive when only v-qPCR is used (Figure 6.7). That suggests that about 0.2 log units of detected live cells were overestimated, if only v-qPCR method is used. A reduction in the culturable *Escherichia coli* number higher than 2.4 log units over 15 minutes was determined by culture, which implies a reduction of more than 97.2% of the control cell number. When the determination was made by qPCR, a reduction of 73.7% was achieved, whereas if it was performed by v-qPCR it reached the 94.3%. If the k&v-qPCR approach was applied, the reduction in the

concentration of *Escherichia coli* was 96.5%, which was closer to the plate counts. When samples were treated with chlorine for 30 min or more, viable qPCR techniques agreed with culture technique.

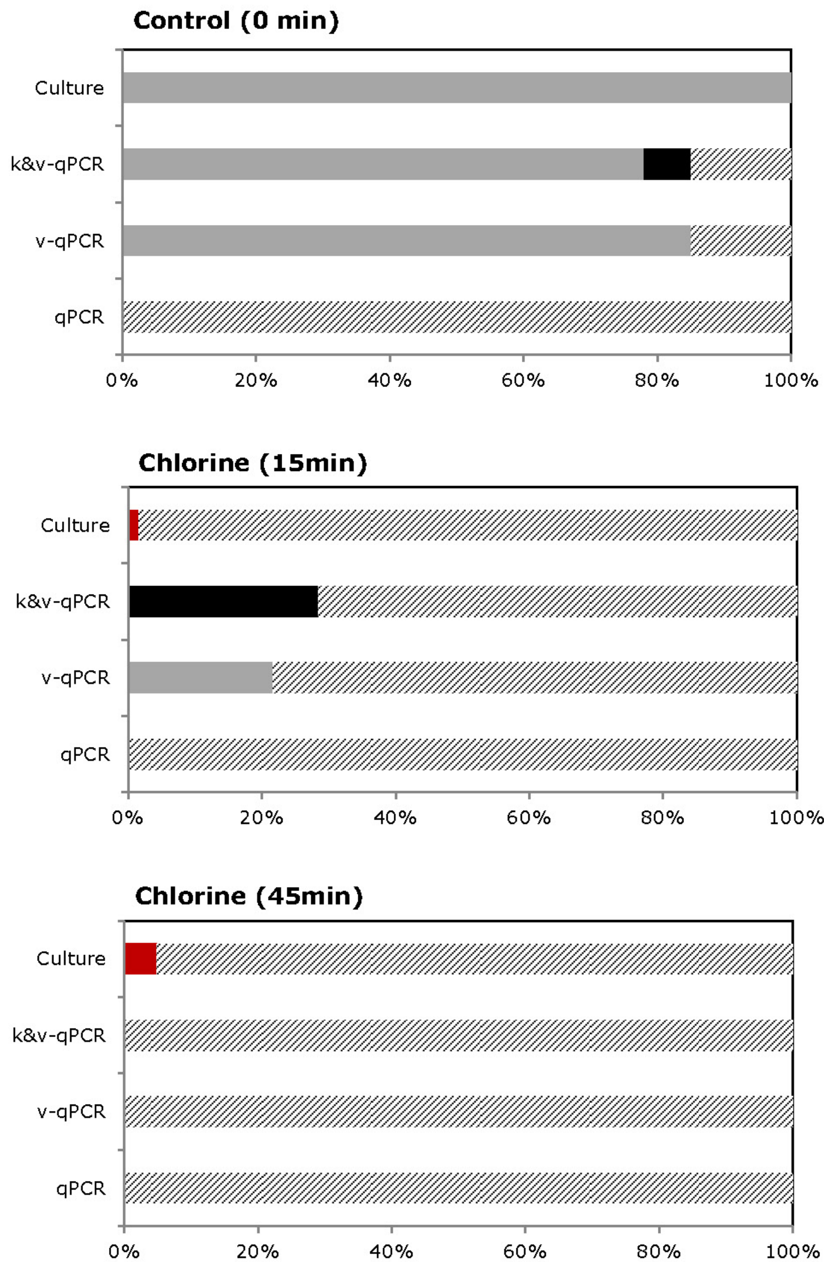


Figure 6.7. Presence of *Escherichia coli* in reclaimed water with chlorine disinfection *in situ* samples. Cell number was measured using three qPCR assays: real-time PCR (qPCR); PMA treatment and qPCR (v-qPCR); killing heat treatment and v-qPCR (k&v-qPCR). Gray bars show the result of *Escherichia coli* viable levels after each assay. Black bars show the possible false positive *Escherichia coli* levels after k&v-qPCR. Hatched gray bars represent total *Escherichia coli* cells number for the qPCR assay, and the dead cell number for culture, v-qPCR and k&v-qPCR assays. Red bars represent the culture detection limit (10 CFU/100 mL). Results were obtained from replicate samples.

The results obtained for samples treated with photocatalysis were confusing and suggest that viable PCR techniques were not completely useful in this case. The reductions obtained in the number of culturable cells by plate counts were not comparable to those found using molecular techniques for any sample time, with exception of the results obtained when 30 min of treatment was performed (bolded numbers in Table 6.2).

Table 6.2. Live *Escherichia coli* cell number reduction during photoactivation treatment.

	Live cell number reduction			
time	**qPCR**	**v-qPCR**	**k&v-qPCR**	**Culture**
15	0.26 (45.6)	0.65 (77.5)	0.82 (84.7)	0.94 (88.4)
30	0.49 (67.4)	**1.31 (95.1)**	**1.43 (96.3)**	1.32 (95.2)
45	0.85 (86)	0.73 (81.2)	0.82 (84.7)	2.25 (99.4)
60	0.56 (72.3)	0.79 (83.4)	0.90 (87.5)	2.38 (100)

Note: The numbers out and in parentheses represent the live cell number reduction in log units and percentage, respectively.

6.4. Discussion

Modifications of the PCR technique involving pretreatment of samples with EMA or PMA prior to DNA extraction have been reported to differentiate viable and dead microbial cells (Nocker and Camper, 2006; Nocker et al., 2006; Nogva et al., 2003). Although membrane integrity is an incomplete criterion for cell viability, the v-PCR approach has received positive evaluation in several publications (Agustí et al., 2010; Bae and Wuertz, 2009; Delgado Viscogliosi et al., 2009; Fittipaldi et al. 2010; Fittipaldi et al., 2011; Rawsthorne et al., 2009). The v-PCR approach is viewed as an important step towards the ability to study live cells using PCR based methods (Nocker and Camper, 2009). In particular, the v-PCR approach has the potential to substantially improve the data on waterborne exposures to several microorganisms (Brescia et al., 2009; Gedalanga and Olso, 2009; Parshionikar et al., 2010; Singh et al., 2013), on disinfection efficacy evaluations (Agustí et al., 2013; Nocker et al., 2007b; Sanchez et al., 2013, Wahman et al., 2009) and enhance the validity of human risk assessment by DNA-based detection methods (Elizaquível et al., 2013; Xing-long et al., 2013). A drawback, however, is that the principle is based on membrane integrity as a viability criterion (Nocker and Camper, 2009). The method was reported as not useful for monitoring the killing efficacy by other inactivation mechanisms that do not directly target the cell membrane, like ultraviolet light irradiation (Nocker et al., 2007b) and it will produce biases in samples that contain viable cells with reversibly damaged membranes. These issues were observed in this study and illustrate the need for critical discussions so that the method can be further improved.

Loss of DNA amplification was observed for both disinfection treatments, chlorination and photocatalysis, when *Bacteroides* spp. and *Escherichia coli* determination assay was performed. It was higher when chlorine (1 mg/L) was used as disinfectant agent. It could indicate that these disinfectant agents can cause DNA damage. Curiously, it was not observed when the *Legionella* detection assay was done using higher concentrations of chlorine (3 mg/L). One reason could be that, as assays were performed in different moments, the organic matter content in the water was different (Westerhoff and Mash, 2002; Yee et al., 2006). Moreover, *Legionella* could be more resistant to chlorination than *Escherichia coli* and *Bacteroides* spp.

For chlorination assays, the viable qPCR techniques -v-qPCR and k&v-qPCR- have shown to be useful. It is not so clear for photocatalysis treatment though. Photocatalysis seems to affect membrane integrity only after 30 min of treatment. In this case, as was suggested by Nocker et al. (2007b) for UV treatment, membrane damage could be an indirect consequence of general cell deterioration. Nocker et al. (2007b) observed that only long UV

exposures exceeding 45 min resulted in increasingly red staining of PMA-treated cells and presumed membrane damage. However, in the study addressed herein, after 45 and 60 min of photocatalysis treatment, the results obtained for qPCR and v-qPCR techniques have not shown important differences. Therefore, further studies are necessary to solve this issue.

Some possible false-negative results were observed when *Escherichia coli* detection study was performed. It is possible that PMA in the concentration used can enter into live cells producing false-negative results. This aspect was also pointed out for different bacteria in previous studies (Kralik et al., 2010; Loozen et al., 2011; Yañez et al., 2011). It seems advisable to determine optimal dye concentrations that efficiently inhibit DNA amplification from membrane-compromised cells, while not affecting signals from intact cells (Fittipaldi et al., 2012). PMA could be used in lower concentrations, such as 10 µM (Nkuipou-Kenfack et al., 2013). It is also probable that the DNA-intercalating dye accessed to DNA in live cells with reversibly damaged membranes or sublethally injured cells; these cells are likely to be present in environmental samples and could recover under adequate conditions. Shi et al. (2011) have indicated that a short incubation of injured *Escherichia coli* cells in recovery medium could potentially minimize the underestimation of live cells and, therefore, the discrepancy between culture and v-qPCR technique. Cytotoxicity studies using non-stressed and stressed cells may be useful to reduce false-negative results (fraction *a* in our proposed methodology).

In the present study, the v-qPCR approach resulted in some cases in a positive DNA amplification signal, when it was used with theoretically dead cells. This outcome suggests that v-qPCR yields false-positive results. Similar results have been observed with other bacteria in previous reports (Bae and Wuertz, 2009; Chang et al., 2010; Kralik et al., 2010) and also presented in Chapter 5 of this dissertation. We noted that the presence of a high number of dead cells is one possible reason for false-positive detection signals, and other researchers have reached similar conclusions (Wang et al., 2009). The false-positive results can be reduced by the optimization of the PMA method with the modification of variables such as PMA concentration, incubation time, incubation temperature, light source, distance from light source, light exposure time, and amplicon length. However, as has been illustrated in the introduction, there may be many factors that could cause significant biases in the application of v-PCR.

The above named limitations in the current v-PCR method led us to seek methods to better estimate the number of viable cells, excluding false positive signals, to improve the ability to assess survival of target organisms exposed to environmental stress and disinfection. Our results also emphasize the need for focusing on the sources of false-positives and false-negatives in future research. Understanding how these sources of error can be experimentally mitigated is ultimately necessary, in order to obtain a better understanding of the samples complexity. Moreover, addressing these errors will ensure a wider acceptance of nucleic acid based methods to assess the viability of target pathogens from environmental samples.

6.5. Conclusion

A realistic approach for assessing the minimum number of viable cells from undefined environmental samples was created based on three qPCR assays. This approach was tested and the bias due to false-positives has been estimated. The feasibility of the approach to assess the minimum number of viable cells with PMA has been validated, at least for these samples, this particular microbial targets, and cell damage caused by disinfection. Although, under the studied conditions, the observed false-positive results did not imply big differences in terms of log units, they can lead to erroneous decisions. For example, during disinfection treatment, longer disinfection times than necessary might be used. Moreover, false-positive fractions can be more important when other killing treatments will be used, or environmental samples without disinfection treatment will be analyzed.

The ideal scenario in most applications of microbial diagnostics is that only live cells are detected. Nevertheless, as Hammes et al. (2011) mentioned before, it is highly unlikely that any 'golden bullet' viability assessment method exist due to the heterogeneous nature of microbial life. The PMA approach is an important step forward in the quantification of live cells by DNA detection based methods. However, as it was addressed in this study and in a recently published review (Fittipaldi et al., 2012), the efficiency of the v-qPCR technique depends on a complex set of parameters which include: dye concentration, the microbial species, cell concentration, the ratio between live and dead cells, the length of the PCR amplicon, and potentially the sequence of the targeted DNA, the turbidity, pH, and salt concentration of the sample, the incubation temperature, and the light source. All factors and their impact on v-qPCR require further examination and discussion in order to improve the method, so not erroneous results are obtained when this approach is applied, especially to environmental samples. The herein outlined k&v-qPCR approach is a step forward to provide a more objective data regarding the number of live microbes and also offers a better understanding of microbial dynamics in complex matrices like wastewater.

6.6. References

AFNOR XP T90-471. 2006. Détection et quantification des *Legionella* et/ou *Legionella pneumophila* par concentration et amplification génique par reaction de polymérisation en chaîne (PCR). Normalization Française.

Agusti, G., Codony, F., Fittipaldi, M., Adrados, B., Morató, J. 2010. Viability determination of *Helicobacter pylori* using propidium monoazide quantitative PCR. Helicobacter, 15, pp. 473–476.

Agusti, G., Fittipaldi, F., Morató, J., Codony, F. 2013. Viable quantitative PCR for assessing the response of *Candida albicans* to antifungal treatment. Appl. Microbial. Biotechnol., 97, pp. 341-349.

Andorrà, I., Esteve-Zarzoso, B., Guillamón, J. M., Mas, A. 2010. Determination of viable wine yeast using DNA binding dyes and quantitative PCR. Int. J. Food Microbiol., 144, pp. 257-262.

Bae, S., Wuertz, S. 2009. Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with propidium monoazide. Appl. Environ. Microbiol., 75, pp. 2940-2944.

Banihashemi, A, Van Dyke, M.I., Huck, P.M. 2012. Long-amplicon propidium monoazide-PCR enumeration assay to detect viable *Campylobacter* and *Salmonella*. J. Appl. Microbiol., 113, pp. 863-873.

Behets, J., Declerck, P., Delaedt, Y., Creemers, B., Ollevier, F. 2007. Development and evaluation of a Taqman duplex real-time PCR quantification method for reliable enumeration of *Legionella pneumophila* in water samples. J. Microbiol. Methods, 68, pp. 137-144.

Bolton, P.H., Kearns, D.R. 1978. Spectroscopic properties of ethidium monoazide: a fluorescent photoaffinity label for nucleic acids. Nucleic acids research, 5, pp. 4981-4903.

Brescia, C.C., Griffin, S.M., Ware, M.W., Varughese, E.A., Egorov, A.I., Villegas, E.N. 2009. *Cryptosporidium* propidium monoazide-PCR, a molecular biology-based technique for genotyping of viable *Cryptosporidium* oocysts. Appl. Environ. Microbiol., 75, pp. 6856-6863.

Cawthorn, D.M., Witthuhn, R.C. 2008. Selective PCR detection of viable *Enterobacter sakazakii* cells utilizing propidium monoazide or ethidium bromide monoazide. J. Appl. Microbiol., 105, pp. 1178-1185.

Chang, B., Taguri, T., Sugiyama, K., Amemura-Maekawa, J., Kura, F., Watanabe, H. 2010. Comparison of ethidium monoazide and propidium monoazide for the selective detection of viable *Legionella* cells. Jpn. J. Infect. Dis., 63, pp. 119-123.

Chen, N.T., Chang, C.W. 2010. Rapid quantification of viable legionellae in water and biofilm using ethidium monoazide coupled with real-time quantitative PCR. J. Appl. Microbiol., 109, pp. 623-634.

Delgado Viscogliosi, P., Solignac, L., Delattre, J.M. 2009. Viability PCR, a culture-independent method for rapid and selective quantification of viable *Legionella pneumophila* cells in environmental water samples. Appl. Environ. Microbiol., 75, pp. 3502-3512.

Desfossés-Foucault, E., Dussault-Lepage, V., Le Boucher, C, Savard, P., LaPointe, G., Roy, D. 2012. Assessment of probiotic viability during Cheddar cheese manufacture and ripening using propidium monoazide-PCR quantification. Front. Microbio., 3:350. doi: 10.3389/fmicb.2012.00350.

Dinu, L.D., Bach, S. 2013. Detection of viable but non-culturable *Escherichia coli* O157:H7 from vegetable samples using quantitative PCR with propidium monoazide and immunological assays. Food Control, 31, pp. 268-273.

Elizaquível, P., Aznar, R., Sánchez, G. 2013. Recent developments in the use of viability dyes and quantitative PCR in the food microbiology field. J. Appl. Microbiol., doi: 10.1111/jam.12365.

E.Z.N.A.® Tissue DNA Kit Handbok. 2012. Cultured cells spin protocol, pp. 12-15. Available in: http://www.omegabiotek.com.

Fittipaldi, M., Rodriguez, N. J., Codony, F., Adrados, B., Peñuela, G., Morató, J. 2010. Discrimination of infectious bacteriophage T4 virus by propidium monoazide real-time PCR. J. Virol. Methods, 168, pp. 228-232.

Fittipaldi, M., Pino Rodriguez, N.J., Adrados, B., Agustí, G., Peñuela, G., Morató, J. 2011. Discrimination of viable *Acanthamoeba castellani* trophozoites and cysts by propidium monoazide real-time Polymerase Chain Reaction. J. Eukaryot. Microbiol. 58, pp. 359-364.

Fittipaldi, M., Nocker, A., Codony, F. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J. Microbiol. Methods, 91, pp. 276-289.

Flekna, G., Stefanic, P., Wagner, M., Smulders, F.J., Mozina, S.S., Hein, I. 2007. Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. Res. Microbiol., 158, pp. 405–412.

Gedalanga, P.B., Olson, B.H. 2009. Development of a quantitative PCR method to differentiate between viable and nonviable bacteria in environmental water samples. Appl. Microbiol. Biotechnol., 82, pp. 587-596.

Graiver, D.A., Saunders, S.E., Topliff, C.L., Kelling, C.L., Bartelt-Hunt, S.L. 2009. Ethidium monoazide does not inhibit RT-PCR amplification of nonviable avian influenza RNA. J. Virol. Methods, 164, pp. 51-54.

Hammes, F., Berney, M., Egli, T., 2011. Cultivation-independent assessment of bacterial viability. Adv. Biochem. Engin. Biotechnol., 124, pp. 123-150.

Heijnen, L., Medema, G. 2006. Quantitative detection of *E. coli*, *E. coli* O157 and other shiga toxin producing *E. coli* in water samples using a culture method combined with real-time PCR. J. Water Health., 4, pp. 487-498.

Kaushik, R., Balasubramanian, R. 2013. Discrimination of viable from non-viable Gram-negative bacterial pathogens in airborne particles using propidium monoazide-assisted qPCR. Sci. Tot. Environmen., 449, pp. 237-243.

Kim, S.Y., Go, G. 2012. Using propidium monoazide to distinguish between viable and nonviable bacteria, MS2 and murine norovirus. Lett. Appl. Microbiol., 55, pp. 182-188.

Kobayashi, H., Oethinger, M., Tuohy, M.J., Hall, G.S., Bauer, T.W. 2009. Unsuitable distinction between viable and dead *Staphylococcus aureus* and *Staphylococcus epidermis* by ethidium bromide monoazide. Lett. Appl. Microbiol., 48, pp. 633-638.

Kralik, P., Nocker, A., Pavlik, I. 2010. *Mycobacterium avium* subsp. *paratuberculosis* viability determination using F57 quantitative PCR in combination with propidium monoazide treatment. Int. J. Food Microbiol., 141, S80-S86.

Layton, A., McKay, L., Williams, D., Garrett, V., R., Sayler, G. 2006. Development of *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl. Environ. Microbiol., 72, pp. 4214–4224.

Loozen, G., Boon, N., Pauwels, M., Quirynen, M., Teughels, W. 2011. Live/dead real time polymerase chain reaction to assess new therapies against dental plaque-related pathologies. Mol. Oral Microbiol., 26, pp. 253-261.

LØvdal, T., Hovda, M.B., Björkblom, B., MØller, S.G. 2011. Propidium monoazide combined with real-time quantitative PCR underestimates heat-killed *Listeria innocua*. J. Microbiol. Methods, 85, pp. 164-169.

Luo, J.F., Lin, W.T., Guo, Y. 2010. Method to detect only viable cells in microbial ecology. Appl. Microbiol. Biotechnol., 86, pp. 377-384.

Meng, X.C., Pang, R., Wang, C., Wang, L.Q. 2010. Rapid and direct quantitative detection of viable bifidobacteria in probiotic yogurt by combination of ethidium monoazide and real-time PCR using a molecular beacon approach. J. Dairy Res., 77, pp. 498-504.

Nam, S., Kwon, S., Kim, M.J., Chae, J.C., Jae Maeng, P., Park, J.G., Lee, G.C. 2011. Selective detection of viable *Helicobacter pylori* using ethidium monoazide or propidium monoazide in combination with real-time polymerase chain reaction. Microbiol. Immunol., 55, pp. 841-846.

Nkuipou-Kenfack, E., Engel, H., Fakih, S., Nocker, A. 2013. Improving efficiency of viability-PCR for selective detection of live cells. J. Microbiol. Methods, 93, pp. 20-24.

Nocker, A., Camper, A.K. 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl. Environ. Microbiol., 72, pp. 1997-2004.

Nocker, A., Camper, A.K. 2009. Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. FEMS Microbiol. Lett., 291, pp. 137–142.

Nocker, A., Cheung, C.Y., Camper, A.K. 2006. Comparison of propidium monoazide and ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Methods, 67, pp. 310–320.

Nocker, A., Sossa-Fernandez, P., Burr, M.D., Camper, A.K. 2007a. Use of propidium monoazide for live/dead distinction in microbial ecology. Appl. Environ. Microbiol., 73, pp. 5111–5117.

Nocker, A., Sossa, K.E., Camper, A.K., 2007b. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. J. Microbiol. Methods, 70, pp. 252-260.

Nocker, A., Mazza, A., Masson, L., Camper, A.K., Brousseau, R. 2009. Selective detection of live bacteria combining propidium monoazide sample treatment with microarray technology. J. Microbiol. Methods, 76, pp. 253-261.

Nogva, H.K., Dromtorp, S.M., Nissen, H., Rudi, K. 2003. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5′-nuclease PCR. BioTechniques, 810, pp. 812–813.

Pan, Y., Breidt, F. 2007. Enumeration of viable *Listeria monocytogenes* cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. Appl. Environ. Microbiol., 73, pp. 8028-8031.

Parshionikar, S., Laseke, I., Fout, G.F. 2010. Use of propidium monoazide in reverse transcriptase PCR to distinguish between infectious and noninfectious enteric viruses in water samples. Appl. Environ. Microbiol., 72, pp. 4318-4326.

Pisz, J.M., Lawrence, J.R., Schafer, A.N., Siciliano, S.D. 2007. Differentiation of genes extracted from nonviable versus viable micro-organisms in environmental samples using ethidium monoazide bromide. J. Microbiol. Methods, 71, pp. 312-318.

Rasmussen, R. 2001. Rapid cycle real-time PCR, methods and applications, quantification on the LightCycler. Meuer, S., Wittwer, C., Nakagawara, K., eds., Springer Press, Heidelberg, Germany, pp. 21–34.

Rawsthorne, H., Dock, C.N., Jaykus, L.A. 2009. PCR-Based method using propidium monoazide to distinguish viable from nonviable *Bacillus subtilis* spores. Appl. Environ. Microbiol., 75, pp. 2936-2939.

Rudi, K., Moen, B., Drømtorp, S.M., Holck, L. 2005. Use of ethidium monoazide and PCR in combination for quantification of viable and dead cells in complex samples. Appl. Environ. Microbiol., 71, pp. 1018-1024.

Sanchez, G., Elizaquivel, P., Aznar, R. 2012. Discrimination of infectious hepatitis A viruses by propidium monoazide real time RT-PCR. Food Environ. Virol., 4, pp. 21-25.

Sanchez, M.C., Marín, M.J., Figuero, E., Llama-Palacios, A., León, R., Blanc, V., Herrera, D., Sanz, M. 2013. Quantitative real-time PCR combined with propidium monoazide for the selective quantification of viable periodontal pathogens in an in vitro subgingival biofilm model. J. Periodontal Res., doi: 10.1111/jre.12073.

Schnetzinger, F., Pan, Y., Nocker, A. 2013. Use of propidium monoazide and increased amplicon length reduce false-positive signals in quantitative PCR for bioburden analysis. Appl. Microbiol. Biotechnol., 97, pp. 2153-2162.

Shi, H., Xu, W., Luo, Y., Chen, L., Liang, Z., Zhou, X., Huang, K. 2011. The effect of various environmental factors on the ethidium monoazide and quantitative PCR method to detect viable bacteria. J. Appl. Microbiol., 111, pp. 1194-1204.

Shi, H., Xu, W., Trinh, Q., Luo, Y., Liang, Z., Li, Y., Huang, K. 2012. Establishment of a viable cell detection system for microorganisms in wine based on ethidium monoazide and quantitative PCR. Food Control, 27, pp. 81-86.

Singh, G., Vajpayee, P., Bhatti, S., Ronnie, N., Shah, N., McClure, P., Shanker, R. 2013. Determination of viable *Salmonellae* from potable and source water through PMA assisted qPCR. Ecotoxicol. Environ. Saf., 93, pp. 121-127. Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Takade, A., Yoshida, S. 2007. Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria. Microbiol. Immunol., 51, pp. 763–775.

Soejima, T., Minami, J., Yaeshima, T., Iwatsuki, K. 2012. An advanced PCR method for the specific detection of viable total coliform bacteria in pasteurized milk. Appl. Microbiol. Biotechnol., 95, pp. 485-497.

Varma, M., Field, R., Stinson, M., Rukovets, B., Wymer, L., Haugland, R. 2009. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. Water Res., 43, pp. 4790-4801.

Vesper, S., McKinstry, C., Hartmann, C., Neace, M., Yoder, S., Vesper, A. 2008. Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). J. Microbiol. Methods, 72, pp. 180-184.

Wagner, A.O., Malin, C., Knapp, B.A., Illmer, P. 2008. Removal of free extracellular DNA from environmental samples by ethidium monoazide and propidium monoazide. Appl. Environ. Microbiol., 74, pp. 2537–2539.

Wahman, D.G., Wulfeck-Kleier, K.A., Pressman, J.G. 2009. Monochloramine disinfection kinetics of *Nitrosomonas europaea* by propidium monoazide quantitative PCR and Live/dead BacLight methods. Appl. Environ. Microbiol., 75, pp. 5555-5562.

Wang, S., Levin, R.E. 2006. Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. J. Microbiol. Methods, 64, pp. 1-8.

Wang, L., Li, Y., Mustapha, A. 2009. Detection of viable *Escherichia coli* O157:H7 by ethidium monoazide real-time PCR. J. Appl. Microbiol., 107, pp. 1719-1728.

Westerhoff, P., Mash, H. 2002. Dissolved organic nitrogen in drinking water supplies: a review. J. Water SRT-Aqua, 51, pp. 415-448.

Willenburg, E., Divol, B. 2012. Quantitative PCR: An appropriate tool to detect viable but not culturable *Brettanomyces bruxellensis* in wine. Int. J. Food Microbiol., 160, pp. 131-136.

Xing-long, X., Cong-cong, L., Yang, Q., Yi-gang, Y., Hui, W. 2013. Molecular monitoring of *Escherichia coli* O157: H7 sterilization rate using qPCR and propidium monoazide treatment. Lett. Appl. Microbiol., 56, pp. 333-339.

Yang, Y., Wan, C., Xu, H., Lai, W., Xiong, Y., Xu, F., You, X., Xu, H., Aguilar, Z.P., Sun, J., Wei, H. 2012. Development of a multiplexed PCR assay combined with propidium monoazide treatment for rapid and accurate detection and identification of three viable *Salmonella enteric* serovars. Food Control, 28, pp. 456-462.

Yañez, M.A., Nocker, A., Soria-Soria, E., Múrtula, R., Martinez, L., Catalán, V. 2011. Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. J. Microbiol. Methods, 85, pp. 124-130.

Yee, L.F., Abdullah, P., Ata, S., Ishak, B. 2006. Dissolved organic matter and its impact on the chlorine demand of treated water. Malays. J. Analytical Sci., 10, pp. 243-250.

Chapter 7

Agricultural reuse of reclaimed water: a preliminary microbial study by using culture and viable qPCR methods

Nowadays, with some Mediterranean regions confronting water shortages and being the agriculture sector the biggest water consumer, it is of particular interest to study the effects of reclaimed water use on crops planned for human consumption. In this chapter, a reclaimed water reuse scenario where three different treated municipal wastewater effluents and groundwater were used for raw edible vegetable crop irrigation was studied. Monitoring of vegetables and microbial water quality was performed by using standard culture technique and real-time PCR (qPCR). The three sources of treated wastewater used, encompassed one obtained with secondary treatment and the other two with different disinfection treatment applied *in situ* (chlorination and photocatalysis). The microbial quality of three different crops was inspected after irrigation. The crops selected were lettuce, carrots and string beans. The results showed that the reclaimed water used could be a safe and reliable resource for agriculture if it is managed properly. A comprehensive real-scale study using reclaimed water from other sources is recommended.

7.1. Introduction

Among the different water usages, agricultural is the largest one with a 50-80% of total freshwater consumption (FAO, 2007; Jiménez and Asanoa, 2008; Palese et al., 2009). This high consumption share is in line with its necessity, given that it is also the activity that produces food for human sustenance. Plants, like all other living things, depend on water to a greater or lesser extent for its vital functions.

According to Tilman (1999), the combination of world population increasing combined with eating habits in some developed countries could cause world demand for grain production to increase more than double. Therefore, it is critical that current agricultural practices have to be modified to minimize environmental impacts, especially those associated to water supply.

Given the situation of stress and competition arising from water resources in the world, the existing climate conditions as well as climate changes due to global warming (Kellis et al., 2013), it is difficult to imagine that the use of water for irrigation could be easily duplicated in the coming decades. Therefore, reduction and activities that increase efficiency of water consumption through better water management should be achieved. Furthermore, the lack of sustainable water resources highlights the need of finding alternative water sources. The reuse of reclaimed wastewater for agricultural irrigation appears as an important contribution to water management, especially in areas with limited freshwater resources. In this sense, water reuse is currently being performed in many places of the world (Lazarova and Bahri, 2005; Pedrero et al., 2010).

The European wastewater Directive (91/271/EEC) prohibits the delivery of wastewater effluents to the environment before reducing possible hazards to their minimum. Therefore, the available volume of treated wastewater is continuously increasing (Pedrero, 2010). Besides the saving of freshwater for other uses and the reduction of wastewater discharges to natural water bodies, the potential use of nutrients such as nitrogen and phosphates contained in reclaimed water is other benefit from the irrigation water reuse practices (Asano, 1998; Pedrero, 2010). However, there are also some limitations to the use of reclaimed water for irrigation. The potential public health and environmental risks associated with reclaimed water use is one of the major limitations. Other important challenge is the public acceptance of such water as a resource instead of a waste. Thus, research is needed to reduce persistent uncertainty about the potential adverse effects that may have the use of reclaimed water on human health and the environment.

As discussed in Chapter 2 of this dissertation (section 2.3.5. *Use of reclaimed water in Spain* and section 2.3.6. *Reclaimed water use in Catalonia*), the treated wastewater is widely used in Mediterranean countries alone or mixed with freshwater for agricultural and landscape irrigation. Currently, 4845 hm^3/year of treated wastewater are produced in Spain, with a reuse rate of 10.14% (491.17 hm^3/year) (EPSAR, 2012). In Catalonia, 692 hm^3/year of wastewater are reclaimed, of which 4.8% are reused (EPSAR, 2012). The main uses are for environmental applications, recreational activities, and for agricultural irrigation[1]. In the year 2009, the Agència Catalana de l'Aigua (Catalonian Water Agency) proposed a water reuse program to achieve the 31% annual reuse rate for the year 2015.

The presence of pathogenic microorganisms in reclaimed water used for agricultural irrigation is of major concern, due to the potential health hazards for the exposed human population. The possible routes of exposure to microorganisms from reclaimed water are the consumption of contaminated vegetable crops and the exposure to aerosols if spray irrigation is performed (Alonso et al., 2006; Yates, 1997). Field workers exposure could also occur through inhalation, ingestion, and dermal contact (Clark, 1987). Periodic monitoring to assess water quality is essential to reduce or eliminate the potential public health hazards when reclaimed water is used.

The microbial quality requirements for reclaimed water depend on its end use, and they must be taken into account when selecting the suitable treatment process to be applied. In Spain,

[1]http://acaweb.gencat.cat. Last Access 08/12/ 2013.

reclaimed water quality is regulated by the Royal Decree 1620/2007, which sets out the physico-chemical and microbiological quality requirements for reclaimed water according its end use (for a broader view of the other possible regulations, refer to Chapter 2 section 2.4. *Reclaimed water regulations and guidelines* of this dissertation).

In order to achieve the required quality, wastewater is treated in sewage plants where primary, secondary, and in some cases, tertiary treatments are performed. Tertiary treatment generally includes a disinfection step. It is an essential step for the use of reclaimed water, because it allows for minimizing the direct and indirect risks for the environment, the users, the inhabited surrounding areas of use, and products' consumers whose production process use reclaimed water.

Water chlorination is one of the most frequently used disinfection method in the world (Moghadam and Dore, 2012). The key to its success lies in its reasonable cost, the accessibility, the relatively simple process, the effectiveness as an antimicrobial agent, and its residual effect. This allows, in a fairly simple way, to ensure water safety from production to the time of use (Solsona and Mendéz, 2002). However, a major disadvantage is the formation of chlorine based disinfection by-products, believed to be carcinogenic and/or mutagenic (Crittenden et al., 2005; Lee et al., 2013; Wang et al., 2013). Moreover, high concentrations of residual chlorine in reclaimed water can have a negative impact on plants irrigated with this water (Stevens et al., 2008). Conventional chemical disinfection methods also have other drawbacks related to the inactivation of resistant pathogens such as protozoa (Hoefel et al., 2005; Sunnotel et al., 2010). For these reasons, there exists a need for additional or alternative disinfection processes such as ultraviolet (UV) radiation or advanced oxidation technologies.

One of the well-known advanced oxidation technologies is heterogeneous photocatalysis, which has proven to be effective for the inactivation of microorganisms in water (Cheng et al., 2007; McCullagh et al., 2007; Nakano et al., 2012; Robertson et al., 2005). It also promises to be an environmental friendly technology. Some studies about the use of this technology to disinfect secondary-treated municipal wastewater have been performed at lab or pilot scale (Alvarez et al., 2011; Lydakis-Simantirisa et al., 2010).

Taking the above into account, in this study a secondary-treated wastewater with two different *in situ* disinfection processes, chlorination and heterogeneous photocatalysis, was used for agricultural drip irrigation. The microbial water quality was monitored, as well as its effect on the microbial contamination of three vegetable crops lettuce (aerial herbaceous plant), carrots (subterranean plant), and string beans (aerial plant with fruit).

7.2. Materials and Methods

7.2.1. Experimental design

A pilot system consisting of a disinfection tertiary-treatment and a greenhouse was built and operated in the experimental facilities of the Institut de Recerca i Tecnologia Agroalimentària (IRTA, Institute of Agriculture and Food Research and Technology Caldes de Montbui, Catalonia, Spain) in order to perform this study.

The secondary-treated wastewater used in the experimental trials, was provided by the Caldes de Montbui wastewater treatment plant (WWTP). The WWTP is designed for a population of 30,000 inhabitants and a daily flow rate of 6,000 m^3. It collects wastewater from the town of Caldes de Montbui, which corresponds to a population of 17,019 inhabitants and 161 industrial establishments, with a network of 12.1 km interceptor sewers. The average daily volume of wastewater collected during 2010 was 4,133 m^3, reaching a maximum of 6,230 m^3 and a minimum of 2,750 m^3. The WWTP is of biological nature, encompassing the process line of: water pretreatment, homogenization, primary sedimentation, biological reactors, secondary settling, and discharge of the treated

wastewater to Caldes River. The generated sludge is concentrated in a gravity thickener and dehydrated by centrifuge.[2]

The secondary-treated wastewater was moved into a tanker from the WWTP to the pilot system, where it was stored in a tank (with capacity of about 20 m^3). The tank had a pumping agitation system, which hindered possible stratification and maintained water homogeneity. The agitation was scheduled every 3 hours.

The flow diagram of the disinfection treatment system is depicted in the Figure 7.1. TK-01 tank was used for storage of reclaimed water coming from the WWTP. This water was pumped (B-01) in three tanks (TK-02, TK-03, and TK-04) whose capacity was 250 L. In order to regulate and control the water level in each tank, three solenoid valves (V-01, V-02 and V-06) and a float regulation device were used. Reclaimed water stored in the TK-02 and TK-03 was pumped (P-02 and P-03, respectively) for recirculation, while passing through disinfection treatment (chlorination or heterogeneous photocatalysis, respectively). Three way valves (V-03 and V-04) enabled the use of these water loops for irrigation. The recirculation time for both treatments was 45 min. The water in the TK-04 did not receive any disinfection treatment and was used as positive control. The tank TK-05, with the same features as the previous ones, was fed with groundwater and it was used as negative control. Reclaimed water without any *in situ* treatment and groundwater were also recirculated during 45 min, before their used for crop irrigation. Irrigation schedule, water recirculation, and tanks' levels were all controlled and supervised using a Programmable Logic Controller (PLC).

Chlorination was carried out using an automatic chlorinator (Stenco, Barcelona, Spain), which allowed for maintaining the desired concentration of free chlorine in the water (from 0.5 to 0.8 mg/L) during disinfection procedures. Sodium hypochlorite was used as a disinfectant agent for chlorination process. The free chlorine and pH determinations were performed using a chlorine test kit (AquaMerck® Chlor, Merck, Darmstadt, Germany), and a field multiparameter instrument (Multi 340i, WTW Inc., Germany), respectively.

Disinfection by advanced oxidation technology was performed using photocatalysis, for which a Benrad water purification facility (Gadelius, Stockholm, Sweden) was used. In order to prevent the organic matter deposition on the photocatalysis lamp, reclaimed water passed through a filter (Regaber, ARKAL, 200 microns) prior photocatalysis treatment. This filter allows achieving better disinfection efficiency, since the radiation loss through absorption by organic matter was avoided.

Each crop grown period was of about three months. Vegetable density was set to 9 plants per m^2, 100 plants per m^2, and 5 plants per m^2 for lettuce, carrots and string beans respectively. Environmental edge effects were avoided by harvesting only center grown plants. Drip irrigation (Rain Bird DI®, Madrid, Spain) was applied in a daily basis, with 60% calculated dose of crop evapotranspiration for the first 10 days and 100% of evapotranspiration for the rest of each assay. The irrigation line consisted in polyvinyl chloride (PVC) and polyethylene pipes.

7.2.2. Irrigation water quality monitoring

All four water types were sampled every two weeks. Water samples of 1 L were collected in sterilized bottles. Sodium thiosulfate (30 mg/L) was used to neutralize the chlorine residual effect for chlorinated water. At least ten samples from each water source were sampled and analyzed.

Water microbiological variables were determined by using traditional culture method or qPCR. The "triple approach" viable PCR (k&v-qPCR) protocol has been used to determine the viability of the microorganism target and the feasibility of this technique for environmental samples. This protocol has been depicted on Chapter 6 of this dissertation, and in Fittipaldi et al. (2011).

[2] http://besos.cat/que-fem/sistemes-de-sanejament .Last access 01/24/2011.

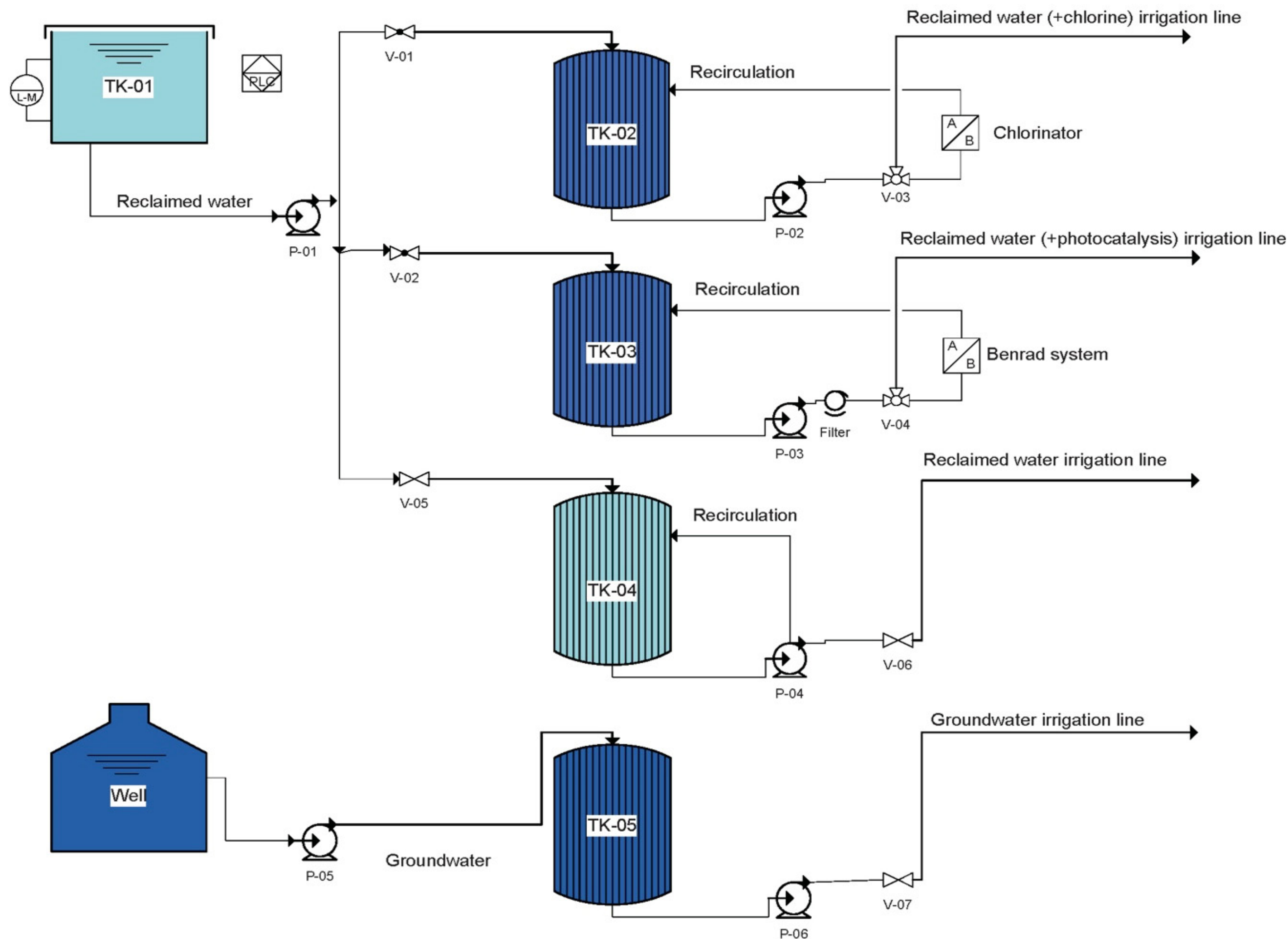


Figure 7.1. Secondary-treated wastewater disinfection pilot system. Four irrigation regimens were used to irrigate vegetable crops: reclaimed water with secondary treatment used directly, reclaimed water with chlorination *in situ*, reclaimed water with photocatalysis treatment *in situ*, and groundwater. PLC: Programmable Logic Controller; TK: tank; P: pump; V: valve.

Determinations of total aerobic bacteria, total coliforms and *Escherichia coli* were performed by conventional culture technique following experimental protocol described in the Chapter 4 of this dissertation (section *4.2.1, Microbiological analysis in water samples – Culture technique*). Enterococci were also determined as was described in the section *4.2.2. Legionella colonization of a lab-scale cooling water using in situ disinfection-Water sample analysis*.

Legionella pneumophila, *Escherichia coli*, *Bacteroides* spp., and *Helicobacter pylori* detections were performed by v-qPCR technique.

Water samples (about 500 mL) were concentrated by centrifugation using a nylon membrane filter (0.45 µm pore, Merck Millipore, Darmstadt, Germany). Filters were resuspended in 10 mL sterile saline solution (0.85% NaCl) into a sterile glass container, and some sterile glass beads (diameter, 5 mm) were added. Cells were detached from the membrane filter by vortex for 60 s, followed by sonication for 3 min in an ultrasound water bath (40 W power, 40 kHz ultrasound frequency; JP Selecta, Barcelona, Spain). The resulting cell suspension was split in three aliquots of 3 mL each. They were concentrated by centrifugation (14,500 rpm for 5 min) using a minicentrifuge (Minispin Plus-Eppendorf, Hamburg, Germany). The supernatant was removed and the pellet was resuspended in 195 µL sterile PBS (1X, pH 7.4) using ultra-transparent 1.5-mL microcentrifuge tubes (VWR, Barcelona, Spain).

PMA (Biotium, Inc., Hayward, California, US) was dissolved in sterile PCR water (Eppendorf, Hamburg, Germany) to create a stock concentration of 2 mM and 10 µL aliquots were stored at -20 ºC in the dark. Five µL of the PMA stock solution were rapidly added to one of the aliquots before prepared. The resultant cell suspension was incubated in agitation (350 rpm, Thermomix, Eppendorf, Hamburg, Germany) for 10 min in the dark at 25 ºC to allow PMA to enter into the cells with compromised membranes. The sample was then photoactivated for 15 min using PhAST blue system (GenIUL, Barcelona, Spain). After photo-induction of cross-linking, cells were pelleted by centrifugation at 14,500 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 200 µL of PBS. The second aliquot was exposed to a killing method that affects membrane integrity before to PMA treatment. The aliquots were exposed to 90 ºC during 15 min. Loss of culturability of heat treated *Escherichia coli* cells was verified by streaking 200 µL of cell suspension and dilutions on Chromocult agar plates (Merck, Darmstadt, Germany) followed by incubation at 37 ºC for 24 h. The third aliquot was also resuspended in 200 µL PBS and it was used for the qPCR assay.

In all cases, DNA was extracted with EZNA tissue DNA purification kit (Omega Bio-Tek, Norcross, USA) according to the manufacturer's instructions (E.Z.N.A.® Tissue DNA Kit Handbook, 2012).

The qPCR protocols used for *Legionella pneumophila*, *Escherichia coli*, and *Bacteroides* spp. detection and quantification were the same than those depicted in the Chapter 6 (section *6.2.4. DNA extraction and quantitative PCR quantification*) of this dissertation.

A previously optimized protocol (Agustí et al., 2010; Pérez et al., 2010) adapted from Kobayashi et al. (2002) was used for *Helicobacter pylori* determination. Nine µL of DNA were mixed with 0.4 µM of each primer, 0.2 µM of FAM labeled probe, and 10 µL of FastStart Taqman Probe Master (Roche, Mannheim, Germany). The amplification conditions were: one step of 95 ºC for 10 min, and then 45 cycles for DNA amplification (95 ºC 15 s and 60 ºC for 1 min). To make a standard curve, the turbidity of a cellular suspension from a positive control of *Helicobacter pylori* (corresponding to a clinical sample isolate), was optically adjusted to 0.2 absorbance units at 600 nm, which correlates to approximately 10^8 CFU/mL. Serial 10-fold dilutions were then carried out from 10^1 to 10^5 CFU/mL, and used as standards. The efficiency of amplification was 100% (Pérez et al., 2010).

For each assay, the threshold cycle (Ct) was determined to quantify each DNA product. Quantification was performed including one or two external standards in each set of PCR experiments. The cell number of each sample was determined using the corresponding standard curve. Each sample was tested in duplicate and mean values were calculated. A negative control, PCR-grade water (Eppendorf, Hamburg, Germany), was included in all assays.

Statistical analyses to calculate mean values and the standard deviations were performed using Microsoft Excel.

During irrigation the monitoring of physico-chemical water quality was determined by considering the following variables:

- Total calcium (determination performed by an external laboratory)
- Total magnesium (determination performed by an external laboratory)
- Total sodium (determination performed by an external laboratory)
- Boron (determination performed by an external laboratory)
- Total organic carbon (TOC) (determination performed by an external laboratory)
- Turbidity
- pH
- Conductivity
- Phosphate (PO_4^{-3})
- Nitrate (NO_3^-)
- Total suspended solids (TSS)

Conductivity and pH were measured using a portable multiparameter instrument (Multi 340i, WTW Inc., Germany). Turbidity, phosphate, and nitrate determinations were performed in the Spectroquant® Nova 60 (Merck, Darmstadt, Germany), following the protocols recommended by the manufacturer. The TSS determinations were performed by filtration through glass fibre filters, dried at 105 °C ± 2 °C, and gravimetric measurement of the mass of the residue retained on the filter, according to the UNE-EN 872:2006.

7.2.3. Irrigated vegetable microbiological quality

Three samples of each vegetable crop -lettuce, carrots and string beans- were collected in sterile plastic bags and taken to the laboratory immediately, keeping them at 4 °C. Only plants grown in the center of the growing area were harvested.

Composite samples of approximately 25 g were prepared, mixing small pieces from different sampled vegetable. These composite samples were transferred to sterile stomacher bags, containing 300 mL of sterile PBS. Samples were turned up and down about ten times and gently rubbed to wash and to release the attached microorganisms on the plant surface (Figure 7.2). The washing step was performed twice. The total wash solution was reserved for making determinations of surface microbial contamination.

For *Escherichia coli*, *Bacteroides* spp., *Helicobacter pylori*, and *Legionella pneumophila* determinations by using qPCR, 100 mL of the homogenization product were concentrated by centrifugation (3,500 rpm for 20 min). Viability PCR was performed using the washing water from lettuce and string beans samples, concentrated again by membrane filtration following the protocol depicted in the section *7.2.2 Irrigation water quality monitoring*.

For plate counts, 10-fold dilutions were prepared from the homogenized sample and the washing water for determination of *Escherichia coli*, total coliforms, total aerobic bacteria and enterococci. All assays were performed in duplicate for each sample.

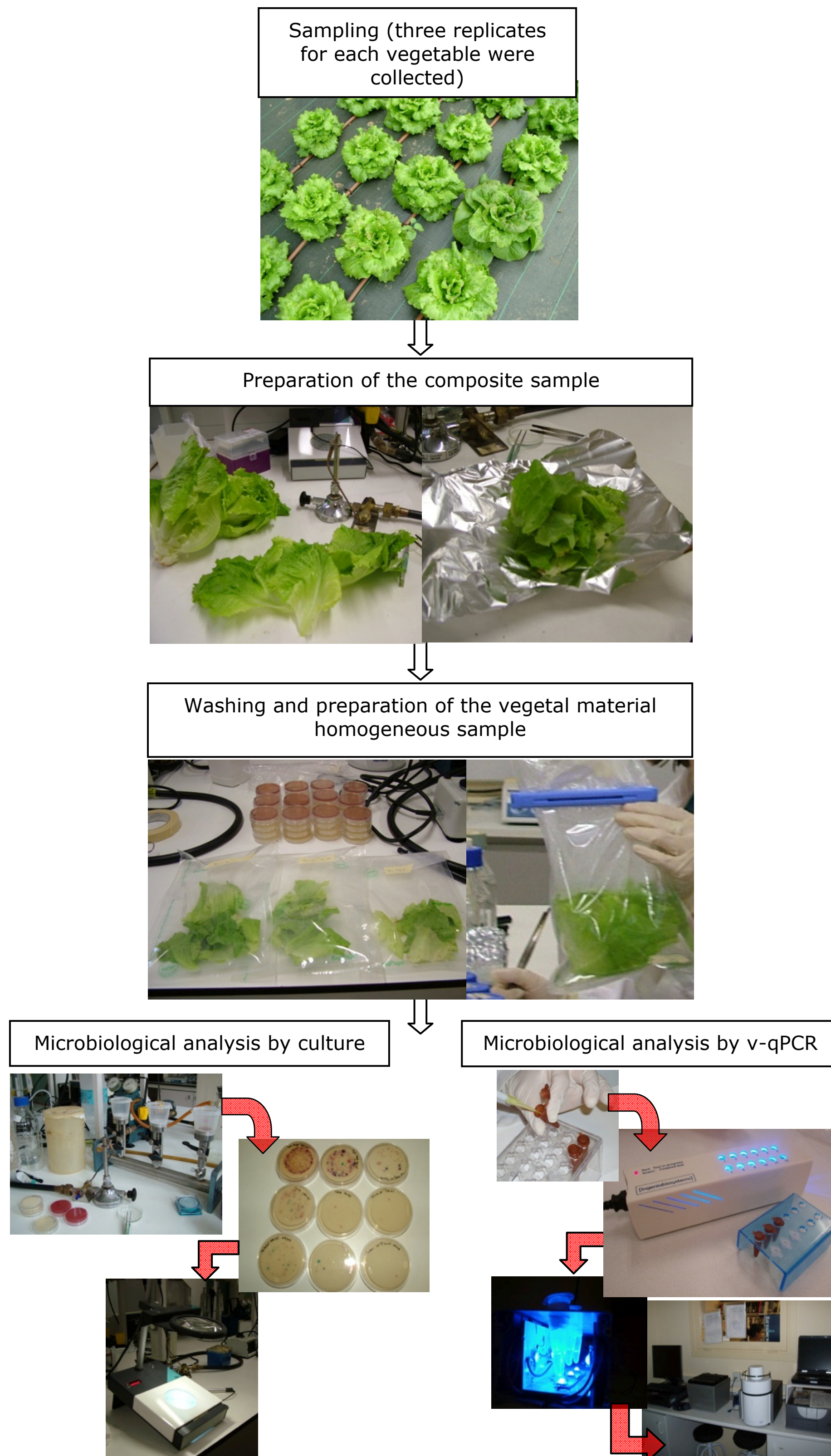


Figure 7.2. Vegetable sampling and analysis process.

7.3. Results

7.3.1. Irrigation water quality monitoring

The average content of free chlorine in the chlorination tank was 0.88 mg/L, while the average concentration at the irrigation point was 0.17 mg/L. Infrequently, there have been some variation in the chlorine concentration, due to the erratic dosage of sodium hypochlorite caused mainly by the sample pH changes. Some of these problems could be produced due to the presence of excessive organic matter, caused by the entry of reclaimed water from the storage tank, when the level of water was low.

Regarding to the photocatalysis system, there were no operational problems, but as in the case of chlorination, there were some difficulties associated to the organic matter presence into the system. A thorough cleaning and flushing of the system solved all the problems.

The results from the physic-chemical and microbiological water quality determinations are summarized in Table 7.1 and Table 7.2, respectively. For calcium, magnesium, sodium, boron, SAR, phosphates and nitrates, significant differences ($p<0.05$) were found between groundwater and the different reclaimed waters. The concentrations of sodium, boron, phosphates, and the SAR values were higher in the reclaimed water with and without disinfection. Calcium, magnesium, and nitrates concentrations were higher in groundwater.

Regarding the microbiological quality of the water samples, for most of the analyzed microorganisms - *Escherichia coli*, total coliforms, enterococci and total aerobic bacteria - significant differences were found between the secondary-treated wastewater and the other types of water sources ($p<0.05$). Non-significant differences were observed between the results obtained from the reclaimed water with photocatalysis or chlorine treatment and groundwater. Also non-significant differences were detected between the effluents from the two different disinfection treatments applied *in situ*.

Table 7.1. Physico-chemical quality of the different water sources.

Parameters	Secondary treated WW	Groundwater	Photocatalysis treated water	Chlorination treated water
Total Calcium (mg/L)	78.00±4.00	196.50± 12.02	81.50±6.36	76.50±0.71
Total Magnesium (mg/L)	28.00±0.00	46.00±1.41	30.00±1.41	28.50±0.71
Sodium (mg/L)	173.67±7.37	32.50±0.71	182.50±19.09	184.00±11.31
Boron (mg/L)	0.27±0.01	0.13±0.00	0.28±0.00	0.27±0.01
TOC (mg/L)	7.09±1.05	<5	6.05±0.24	12.60±5.51
SAR (meq/L)	4.29±0.25	0.54±0.03	4.40±0.53	4.56±0.25
Turbidity (NTU)	0.84±0.13	0.27±0.06	0.72±0.09	0.76±0.20
pH	8.26±0.16	7.87±0.06	8.30±0.14	8.09±0.47
Conductivity (uS/cm)	1358.25±41.78	1374.13±22.94	1365.50±37.45	1475.63±52.57
$PO4^{-3}$ (mg/L)	11.05±1.29	0.51±0.12	12.70±1.55	11.81±1.68
NO_3^- (mg/L)	6.70±1.62	330.74±30.25	7.12±1.35	5.96±1.09
TSS (mg/L)	6.80±2.06	8.80±1.53	5.83±1.35	6.80±1.09

Note: SAR (Sodium Adsorption Ratio); TSS (Total Suspended Solids); TOC (Total Organic Carbon); SD (Standard Deviation); WW (wastewater).

Table 7.2. Microbiological water quality determined by culture.

Microorganisms	Detection	Groundwater	Secondary treated WW	RW (photocatalysis)	RW (chlorination)
Escherichia coli	Positive (%)	10	100	30	30
	Negative (%)	90	0	70	70
	Mean value±SD (CFU/100 mL)	<10	48±76	<10	<10
	Maximum value (CFU/100 mL)	9	260	17	41
Total coliforms	Positive (%)	100	100	100	100
	Mean value±SD (CFU/100 mL)	832±429	3130±374	72±17	80±20
	Maximum value (CFU/100 mL)	875	5680	210	180
Enterococci	Positive (%)	70	100	40	50
	Negative (%)	30	0	60	50
	Mean value ±SD (CFU/100 mL)	<10	31±23	<10	<10
	Maximum value (CFU/100 mL)	20	80	22	36
Total aerobic bacteria	Positive (%)	100	100	100	100
	Mean value ±SD (CFU/mL)	$4.1\ 10^3 \pm 1.8\ 10^3$	$1.4\ 10^5 \pm 1.3\ 10^5$	$1.3\ 10^4 \pm 4.7\ 10^3$	$1.8\ 10^4 \pm 6.2\ 10^3$
	Maximum value (CFU/mL)	$1.9\ 10^4$	$3.5\ 10^5$	$4.0\ 10^4$	$6.4\ 10^4$

Note: Ten samples were analyzed for each water type. WW: wastewater. RW: reclaimed water. SD: standard deviation. Detection limit = 1 CFU/100 mL.

It is also important to note than in many cases the standard deviation was high, due to the great variability of the water quality.

All the secondary-treated wastewater samples tested were culture positive for *Escherichia coli* and most of them (90%) showed a concentration range of 10 to 50 CFU/100 mL. These values are lower than those established by the Royal Decree 1620/2007 for the use of reclaimed water for agricultural irrigation of raw eaten vegetables (quality criterion 2.1; <100 CFU/100 mL). Only one sample (10%) did not meet the established quality criteria, with an average concentration of 260 CFU/100 mL.

For 90% of cases *Escherichia coli* was not detected by culture method (<1 CFU/100mL) in groundwater, while for the remaining 10% count was less than 10 CFU in 100 mL. For reclaimed water treated with photocatalysis, *Escherichia coli* was not detected in 70% of the analyzed samples by plate count, for the 20% of the samples the counts were below 10 CFU/100 mL, and for the remaining 10% the counts were lower than 20 CFU/100 mL. Similar results were found in chlorinated water samples. In this case, the presence of *Escherichia coli* was not detected in 70% of the samples, at 20% of cases the count was lower than 10 CFU/100 mL, and for the other 10% counts resulted to be less than 50 CFU/100 mL.

The presence of *Escherichia coli* was not detected when groundwater and disinfected reclaimed water samples were analyzed by v-qPCR. In the case of secondary-treated wastewater, positive results were obtained for 70% of samples when qPCR was used, while only 20% were positive for v-qPCR (Table 7.3). These results agreed with those obtained by culture, since only one sample was higher than the quality requisite, 100 CFU/100 mL. Regarding the other positive samples, the v-qPCR detection limit was calculated to be 67 CFU/100 mL, and most of the plate count results were near or below it. Thus, a positive sample showed a higher concentration by v-qPCR than by culture procedure.

Table 7.3. *Escherichia coli* quantification in secondary treated wastewater by qPCR with and without PMA.

Samples	***qPCR (CFU/100mL)***	***v-qPCR (CFU/100mL)***	***K&v-qPCR (CFU/100mL)***
1	<Lod	<Lod	<Lod
2	525	<Lod	<Lod
3	283	<Lod	<Lod
4	<Lod	<Lod	<Lod
5	161	<Lod	<Lod
6	275	179	<Lod
7	144	<Lod	<Lod
8	<Lod	<Lod	<Lod
9	85	<Lod	<Lod
10	82	69	<Lod

Lod: limit of detection, 67 CFU/100 mL.

All samples collected from the different water sources were positive when total coliforms detection was performed. The concentration was about 0.5 log and 1.5 log units higher for the secondary-treated wastewater samples than for the groundwater samples and the disinfection effluent samples, respectively. Determinations of fecal coliforms were performed in some samples. The mean values were 33±25 CFU/100 mL for groundwater samples, 263± 82 CFU/100 mL for secondary-treated wastewater samples, 51±18 CFU/100 mL for reclaimed water-photocatalysis samples, and 54±4 CFU/100 mLfor reclaimed water-chlorination samples.

Enterococci counts were below 10 CFU/100 mL for the 40% of the analyzed secondary-treated wastewater samples, whereas for the remaining 60% the counts were below 100 CFU/100 mL.

In 50% of the chlorinated water samples the presence of enterococci was not detected (<1 CFU/100 mL), in a 30% of samples the counts were below 10 CFU/100 mL, and in the other 20% counts were around 40 CFU/100 mL. In the case of photocatalysis, for the 60% of the samples enterococci were not detected, whether in the remaining 40 percent results were below 25 CFU/100 mL.

The 30 percent of the groundwater samples were negative for enterococci; counts were below 10 CFU/100 mL for the 40 percent of samples, and below 25 CFU/100 mL for the remaining 30 percent.

As expected, all samples taken from the different water sources were positive when total aerobic bacteria presence was studied. The concentration was higher for the secondary-treated wastewater samples than for the groundwater samples (2 log units), and disinfection effluents (1 log unit).

Legionella pneumophila was not detected in any of the 40 water samples analyzed -10 of the secondary- treated wastewater, 10 of the reclaimed water with chlorination, 10 of reclaimed water with photocatalysis, and 10 of groundwater. The limit of detection was 533 CFU/L.

Bacteroides spp. detection was negative for groundwater samples, and reclaimed water with *in situ* disinfection, photocatalysis or chlorination. In the case of secondary-treated wastewater, 20% of samples were positive by qPCR without PMA pretreatment, while no sample was positive by v-qPCR. The two positive samples for qPCR showed low concentrations such as 87 and 124 CFU/100 mL. The calculated limit of detection was 67 CFU/100 mL.

The v-qPCR results showed that live *Helicobacter pylori* cells were detected in one secondary-treated wastewater sample. The concentration was approximately 1.5 log units/L, when the triple approach was applied, 4.4 log units when v-qPCR was applied, and 5 log units when only qPCR was used. Regarding the reclaimed water samples, only one chlorinated sample was positive for qPCR. Results below the detection limit (740 CFU/L) were found when viability techniques where performed. In samples treated with photocatalysis, four samples were positive by qPCR in the order of 10^3 to 10^5 CFU/L, two samples were positive by v-qPCR in the order of 10^3 CFU/L, while only one sample was positive when the triple approach was applied (in the order of 10^3 CFU/L). Three groundwater samples showed to be positive for *Helicobacter pylori* by qPCR, but they were under the detection limit when viability techniques were used.

7.3.2. Vegetable microbiological quality

Three different type of vegetables - roots (carrots), aerial herbaceous (lettuce) and aerial plants with fruit (string beans) - irrigated with four different water sources were sampled by triplicate. Their microbial quality was analyzed using culture and qPCR techniques. Furthermore, the microbiological quality of the vegetable wash water samples was also investigated.

Lettuce

Escherichia coli were not detected in any of the vegetable wash water samples or plant material samples analyzed by culture technique. The detection limit was 5 CFU/g for wash water and 12 CFU/g for plant material. Regarding the detection by qPCR, inhibition of amplification was observed when direct samples were analyzed. Thus, decimal dilutions of the purified DNA from wash water samples were performed before qPCR analysis. In the case of DNA from plant material, higher dilutions such as 1/100 and 1/1000 were needed. Only one replica of lettuce irrigated with secondary-treated wastewater was positive for *Escherichia coli,* with a concentration of 4.9 10^5 CFU/g. The limit of detection for the vegetal material analysis was 3.96 10^3 CFU/g, and it was 112 CFU/g for the wash water analysis.

The presence of enterococci was not detected neither for wash water samples nor lettuce samples. The detection limit of the technique was 5 CFU/g and 12 CFU/g for wash water and vegetable material, respectively.

The total coliform count for the vegetable wash water samples was in the order of 2.6 log units per gram of lettuce irrigated with groundwater, 3.7 log units for plants irrigated with secondary-treated wastewater, and between 3.1 and 3.2 log units when chlorination or photocatalysis treated reclaimed water were used for crop irrigation, respectively (Figure 7.3). The reduction achieved in the total coliform population including two vegetable washes was approximately 1.15 log units.

Regarding the total coliform presence in the analyzed vegetable material, the counts were about 2.3 log units over those obtained for the second wash water samples, when vegetables were irrigated with groundwater or secondary-treated wastewater, and about 0.7 log units in the case of vegetable irrigated with reclaimed water obtained by chlorination or photocatalysis disinfection *in situ*, respectively (Figure 7.3).

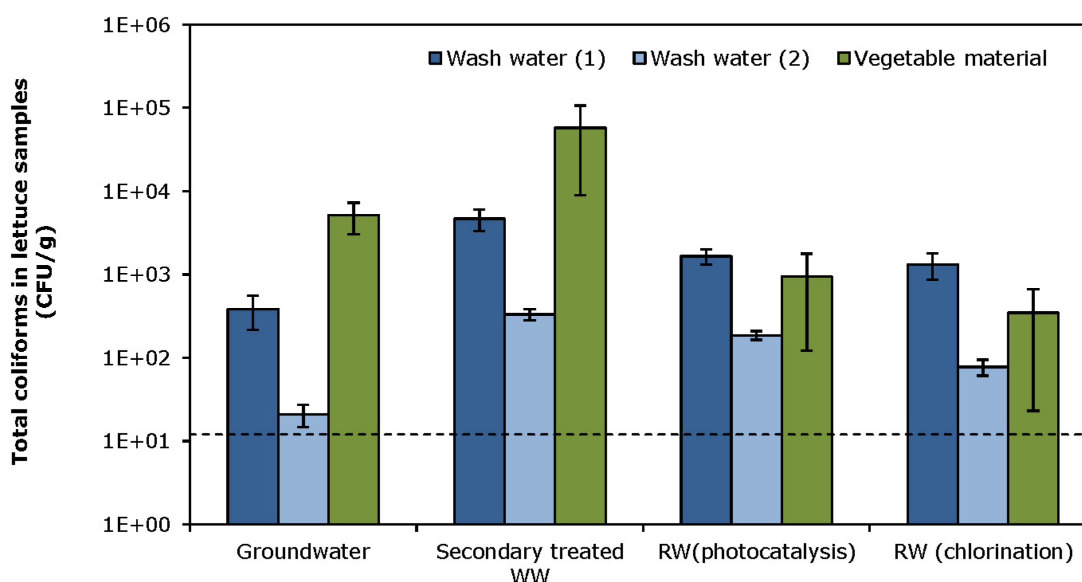


Figure 7.3. Total coliform plate counts for lettuce samples irrigated with waters of four different qualities. The columns represent the mean value from 3 replicates. The error bars represent the standard deviation from three independent assays. The dashed line indicates the limit of detection of the technique: 12 CFU/g for plant material. The detection limit for the wash water was 5 CFU/g. WW: wastewater. RW: reclaimed water.

Taking into account the obtained results from wash water analysis, lower counts were obtained when groundwater was used as irrigation water. Statistical significant differences ($p<0.05$) were found between the results obtained for this type of water and those obtained for the other water used for crop irrigation. Also significant differences ($p<0.05$) were observed between secondary-treated wastewater results and chlorine disinfected water results. Regarding to the vegetable analysis, significant differences ($p<0.05$) were observed between the vegetables irrigated with chlorine treated reclaimed water, and vegetables irrigated with groundwater or secondary treated wastewater. But no significant results were detected between the vegetables irrigated with reclaimed water, both chorine and photocatalysis treated. The high variation between the three analyzed replicates could explain the lack of significant differences between photocatalysis treated reclaimed water and secondary-treated wastewater or groundwater.

As expected, *Bacteroides* spp. were detected in the wash water samples of lettuce irrigated with secondary-treated wastewater by qPCR with and without PMA treatment at concentrations of 111 and 298 CFU/g respectively. Conversely, *Bacteroides* spp. were not detected in the wash water samples of vegetables irrigated with groundwater, nor in those irrigated with disinfected reclaimed water (chlorination or photocatalysis), being the detection limit 47 CFU/g.

Regarding the vegetable processed samples, DNA amplification inhibition was observed when direct samples were used, so 1/100 and 1/1000 dilutions from direct samples were prepared and analyzed. *Bacteroides* spp. determinations were below the limit of detection, $7.9\ 10^3$ CFU/g for all cases.

The amplification of *Helycobacter pylori* by qPCR was also affected by inhibition. Thus, sample dilution of 1/10 for the wash water and 1/100 and 1/1000 for the plant material were performed to overcome it. Two replicates of wash water samples from lettuce plants irrigated with secondary-treated wastewater were positive showing an average concentration of 520 CFU/g, with a detection limit of 260 CFU/g. The qPCR results of the same samples treated with PMA were below the detection limit.

In all cases, aerobic bacterial counts were lower for the plant material than for wash water (Table 7.4). The counts for lettuce samples irrigated with reclaimed water with disinfection were lower those for vegetable samples irrigated with secondary-treated wastewater and groundwater. Significant differences were found ($p<0.05$) between the results obtained for wash water samples from lettuce irrigated with secondary-treated wastewater, and those obtained for vegetable irrigated with other type of water (groundwater, reclaimed water (+chlorine), or reclaimed water (+photocatalysis)).

Regarding the analysis of vegetable material, no significant differences ($p>0.05$) were found between those irrigated with secondary-treated wastewater or groundwater. On the other hand, significant differences were found between them and the vegetables irrigated with disinfected waters. No significant statistical differences were observed between the results obtained for disinfection process both, chlorination, and photocatalysis.

Table 7.4. Total aerobic bacteria in lettuce samples irrigated with water of different quality.

Total aerobic bacteria (CFU/g)		
Irrigation water	**Wash water**	**Vegetable material**
Groundwater	6.07±0.30	5.75±0.75
Secondary-treated WW	6.77±0.21	5.52±0.25
RW (photocatalysis)	6.30±0.38	4.66±0.56
RW (chlorination)	6.39±0.51	4.97±0.45

Note: WW: wastewater. RW: reclaimed water

Carrots

Escherichia coli was detected in the wash water samples by culture (Figure 7.4). The reduction of the bacterial load from wash 1 to wash 2 was about 0.9 log units, being greater in the case of reclaimed water (1.28 log units). The bacterial concentrations detected for samples from the second washing were close to the limit of the detection, 1 CFU/g of vegetable analyzed.

Escherichia coli was not detected on carrot samples by culture, being the detection limit of 5 CFU/g. The determination by qPCR was strongly inhibited for both, wash water and vegetable material samples. Although some dilutions were analyzed this bacterium was not detected neither in wash water samples nor carrot plant material, being the detection limit of 9.26 10^3 CFU/g and 6.94 10^3 CFU/g, respectively.

Significant differences ($p<0.05$) were seen in the results obtained for the first washing, between the secondary-treated wastewater and the other type of waters studied. For the second vegetable washing, the results gathered for the vegetables irrigated with reclaimed water (+ photocatalysis) were significantly different than those found for carrots irrigated with secondary-treated wastewater or groundwater.

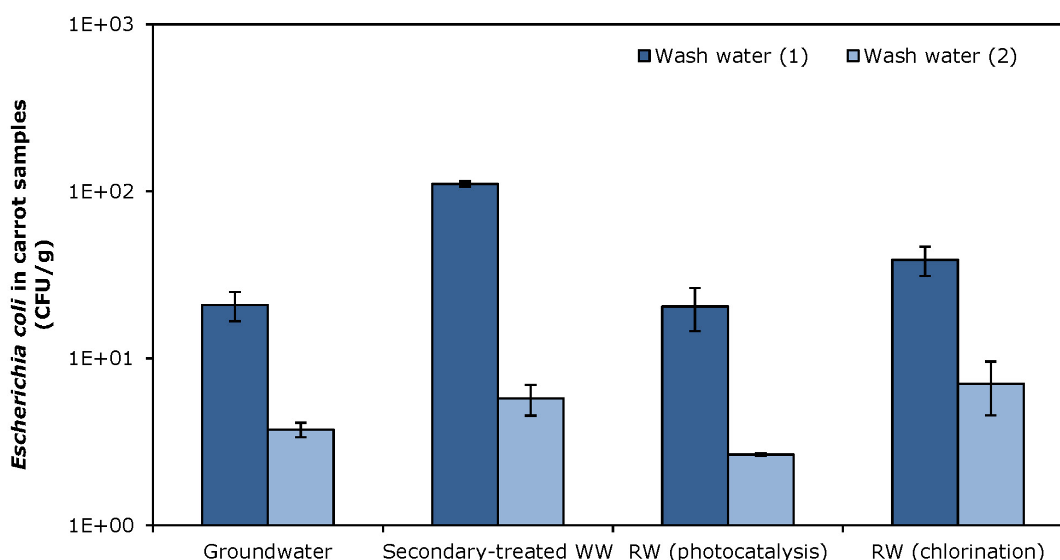


Figure 7.4. *Escherichia coli* plate counts for wash water samples of carrots irrigated with waters of four different qualities. The columns represent the mean value from three replicates. The error bars represent the standard deviation from three independent assays. The detection limit for the wash water was 1 CFU/g. WW: wastewater; RW: reclaimed water.

Enterococci were not detected in any of the samples, wash water or plant material, being the detection limit of the technique for both types of samples 10 CFU/g.

The total coliform counts in the carrot wash water samples were in the order of 3 to 3.5 log units per gram of carrot. An approximate load reduction of 0.85 log unit was achieved for total coliform population, from the first to the second washing (Figure 7.5). In the case of vegetable material, the counts were higher than those obtained for the second washing for the groundwater and the secondary-treated wastewater, in 0.45 and 0.11 log units, respectively. For carrot samples irrigated with chlorinated and photocatalysis treated water, total coliform counts were lower than those detected in the second washing (Figure 7.5).

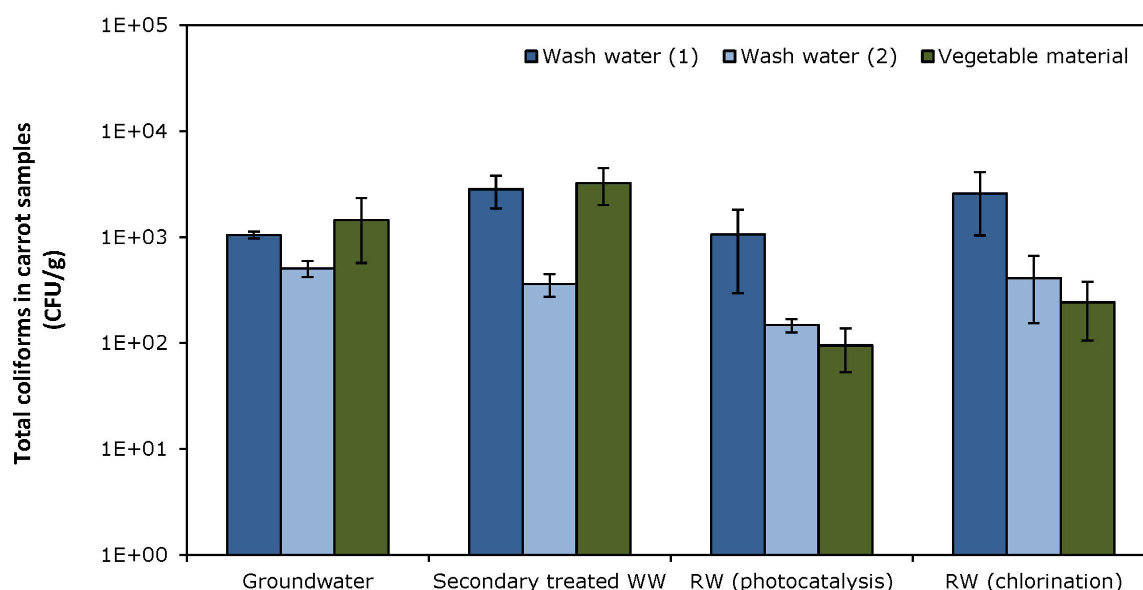


Figure 7.5. Total coliform plate counts for wash water and vegetable samples of carrots irrigated with waters of four different qualities. The columns represent the mean value from three replicates. The error bars represent the standard deviation from three independent assays. WW: wastewater; RW: reclaimed water.

Statistical significant differences ($p<0.05$) were found in the first washing process, between the vegetables irrigated with secondary-treated wastewater and those irrigated with groundwater or photocatalysis treated reclaimed water. On the other hand, no differences were seen between the samples irrigated with the disinfected reclaimed waters. In the second washing, only significant differences were observed between the vegetables irrigated with photocatalysis treated water and those irrigated with secondary-treated wastewater and groundwater.

About the microbial analysis of the vegetable material, significant differences were observed between the carrots irrigated with secondary-treated wastewater or groundwater, and those irrigated with disinfected reclaimed waters. No differences were found between secondary-treated wastewater and groundwater.

Bacteroides spp. and *Helicobacter pylori* were not detected in any of the analyzed samples. The *Bacteroides* and *Helicobacter pylori* limits of detection were 1.7 10^4 CFU/g and 9.26 10^3 CFU/g for the wash water samples, and 1.2 10^4 CFU/g and 6.94 10^3 CFU/g for the vegetable material, respectively.

As in the case of the results obtained for lettuce microbial analysis, heterotrophic bacterial counts for wash water were comparable or even greater than those found in plant material. The counts were in the order of 5.1 to 5.8 log units. No significant differences between the different used irrigation water were found.

<u>*String beans*</u>

Escherichia coli bacteria were not detected in any sample using both, culture and molecular techniques. The limits of detection for wash water samples were 10 CFU/g for culture analysis, and 324 CFU/g for qPCR. In the case of vegetable samples the detection limits were 9 CFU/g and 1.68 10^3 CFU/g for plate count and qPCR, respectively.

Like with lettuce and carrots, inhibition of the DNA amplification was observed and decimal dilutions were done to obtain reliable results.

Enterococci were not detected in any of the samples from the second washing and the plant material analysis, being the limit of detection of 6 CFU/g and 9 CFU/g for wash water and vegetal samples, respectively. However, they were detected in the wash water samples from the first washing, with a mean concentration of 22 CFU/g for string beans irrigated with secondary-treated wastewater and the two disinfected reclaimed waters, and 10 CFU/g for samples irrigated with groundwater.

A total coliform load reduction of about 1 log unit was observed from the first to the second washing (Figure 7.6). Regarding the plant material counts, they were in the order of 3 log units for string beans irrigated with groundwater, and 4 log units for the other type of water used. They were higher than the coliform counts obtained from the analysis of the second washing samples (Figure 7.6). No statistical significant differences were observed between the resulted obtained for the different used irrigation waters. It could be due to the higher variation in the acquired results from the three replicates.

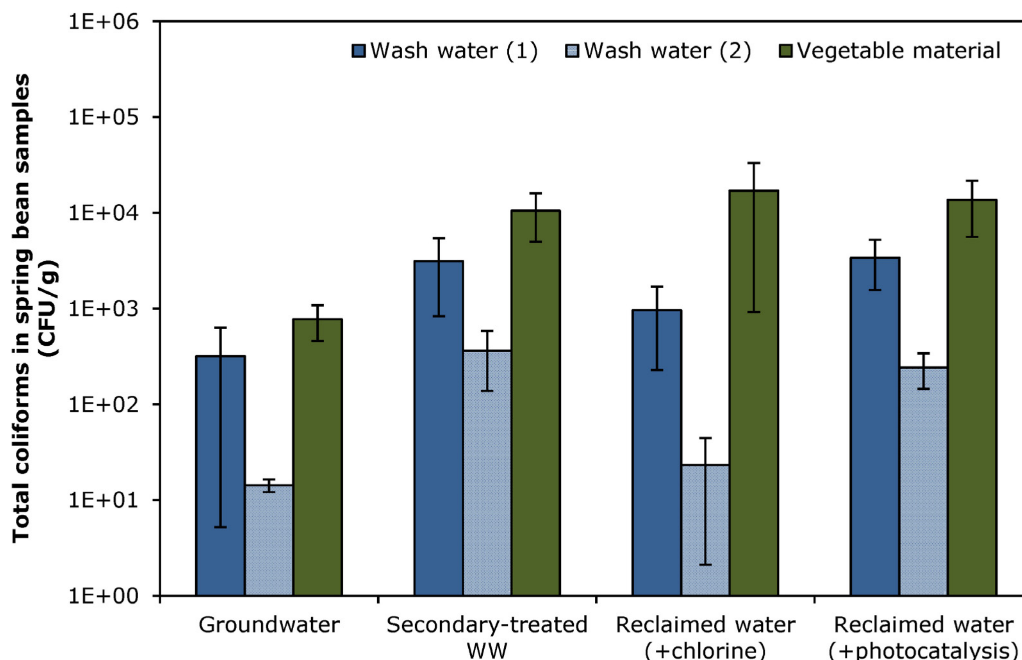


Figure 7.6. Total coliform bacterial plate counts from vegetable and wash water samples of string beans irrigated with waters of four different qualities. The columns represent the mean value from three replicates. The error bars represent the standard deviation from three independent assays. WW: wastewater. The limit of detection was 10 CFU/g for wash water, and 9 CFU/g for plant material samples.

Bacteroides spp. and *Helicobacter pylori* were not detected. The *Bacteroides* and *Helicobacter pylori* limits of detection were 6.4 10^2 CFU/g and 3.6 10^3 CFU/g for the wash water samples, and 2.7 10^3 CFU/g and 1.5 10^3 CFU/g for the vegetable material, respectively.

Heterotrophic bacterial counts were 1 log unit higher for wash water comparing with the plant material. Plate counts were in the order of 6 log units for the wash water samples, and in the order of 5 log units for the vegetable material samples in all cases, except in the case of chlorinated water with 1 log unit lower results. Thus, the aerobic bacteria load of vegetables irrigated with chlorinated water was in the order of 5 and 4 log units for the wash

water, and plant material, respectively. Significant differences between the obtained resulted were not seen.

7.4. Discussion

The microbiological quality of different vegetables - lettuce, carrots and string beans- after being irrigated with water of different microbiological qualities was studied. Also, microbiological monitoring of the irrigation water sources – secondary-treated wastewater, reclaimed water (chlorine, <1mg/L), reclaimed water (photocatalysis), and groundwater- was performed. The microbiological quality of both, the crops and the irrigation water, was determined by using conventional microbiological techniques, such as culture, and molecular biology techniques, such as qPCR and v-qPCR.

The currently in-force Royal Decree (RD 1620/2007), which establishes the legal framework for the treated wastewater reuse in Spain, dictates the water quality requirements (Table 7.5) for the use of reclaimed water in raw eaten crops irrigation.

Table 7.5. Water quality requirements for reclaimed water use for raw eaten vegetable irrigation (RD 1620/2007).

Quality Criteria	*Variable*	*Limit values*
Microbiological	Intestinal nematode parasites	1 egg/10 L
	Escherichia coli	100 CFU/100 mL
	Legionella spp.	1,000 CFU/L*
Physical-Chemical	TSS	20 mg/L
	Turbidity	10 NTU
	EC	3 dS/m
	SAR	6 meq/L
	Boron	0.5 mg/L

*In the case that exist aerosolization risk, for example when sprinkler irrigation is performed.

Taking into account the results obtained in this study from the analysis of physico-chemical quality of the different types of water used in irrigation, both secondary-treated wastewater and disinfected reclaimed waters accomplished with the requirements of the Royal Decree. The analyzed physico-chemical variables were also within the usual range established by Mujeriego (1990) for irrigation water.

It is worth of mention that although the physico-chemical variables values from groundwater analysis were also within the quality requirements, the content of nitrate (NO_3^-) detected in this water was high (330 mg/L). This value exceeds the allowed maximum of 50 mg/L set in the sanitary-technical regulation for the supply and quality control of public drinking water (RD 1138/1990). The Nitrates Directive (91/676/EEC) and the Drinking Water Directive (98/83/EC) established an identic 50 mg/L standard. The high nitrogen content in groundwater may be associated with the intensive use of nitrogenous fertilizers. The consumption of high nitrate amounts in the human diet is dangerous, because this ion can contribute to the formation of carcinogen products (Garbisu et al., 1999; Jaworska, 2005). For this reason, the European Commission has legislated, indicating the maximum levels of nitrates allowed to lettuce grown under cover and in the open air at different times of the year (European Food Safety Authority, 2010). So, nitrates should be an important chemical variable to analyze from vegetable samples.

Microbiological quality of irrigation water

Regarding the microbiological quality of the different water types used in irrigation, secondary-treated wastewater was positive for *Escherichia coli* in 90% of cases, but only 10% of them exceeded the limit of 100 CFU/100 mL established by the RD 1620/2007. This level of *Escherichia coli* is low in comparison with that found in secondary-treated wastewater previously reported in other studies (Gómez et al., 2006; Muñoz et al., 2010; Omar and Barnard, 2010). Levantesi et al (2010) found *Escherichia coli* concentrations between 10^3 and 10^5 CFU/100 mL in secondary effluents from three different European WWTPs. However, it is important to note that the frequency of detection of this microbial indicator reported by the authors was 100%, equal to that observed in this study. Meanwhile, 70% of the samples collected from photocatalysis treated reclaimed water tank and chlorine treated reclaimed water tank were below the limit of detection (<1 CFU/100 mL). For the remaining samples, counts were below the limits established by the Royal Decree. The good quality of on-site treated effluents agreed with values reported in the literature for effluents reclaimed through disinfection process such as chlorination or advanced tertiary treatments (Al-Sa'ed, 2007; Levantesi et al., 2010; Muñoz et al., 2010). For groundwater samples, in the 90% of cases the results were below the limit of detection (<1 CFU/100 ml), as expected, and the other 10% accomplished the established quality requirements. These results also agreed with previously reported *Escherichia coli* levels in groundwater (Levantesi et al., 2010).

The results obtained by molecular techniques were consistent with those obtained by plate counts. Only two secondary-treated samples were positive for *Escherichia coli* viable cell detection, and just one of them showed a concentration higher than those established by the in force regulations in Spain. A positive sample showed higher levels by v-qPCR than by culture procedure. Some possible causes that could help to explain this issue were discussed in the Chapter 3 of this thesis (section 3.4. *Discussion*). One of them is that both techniques detect the presence of cells using different units that are not strictly comparable. Other explanation could be related with the presence of viable but not-culturable cells which are detected by qPCR but not by culture.

Regarding the content of *Legionella* spp., the presence of *Legionella pneumophila* by qPCR resulted to be negative in all the analyzed samples likely because they were not present at high enough levels, being the limit of detection of 533 CFU/L. This is in agreement with the results reported by Shannon et al. (2007). However, the presence of *Legionella* in reclaimed water should be long-term studied as it was pointed out by Huang et al. (2009), whom have demonstrated that *Legionella* are ubiquitous in WWTP in Taiwan, especially if sprinkler irrigation will be applied.

Enterococci are also an important pollution indicator and, therefore, their presence has also been studied in this work. Their resistance to disinfection processes and environmental factors make them correlate strongly to adverse health situations (McLain and Williams, 2012; Pourcher et al., 2007; Salgot et al., 2006). Generally, no indication about the maximum allowable concentration of enterococci is given in international legislation (Palese et al., 2009). In all cases analyzed in this study, the enterococci bacterial counts were lower than the limit imposed for *Escherichia coli* (100 CFU/100 mL) for the local health authorities.

Total aerobic and coliform bacterial counts are usually used to demonstrate the efficiency of disinfection treatments and to have a notion of the microbiological load. Maximum total coliforms counts were in the order of 4 log unit for secondary-treated wastewater, and in the order of 3 log unit for the other used irrigation waters. However, non-significant differences were seen between the different water sources. It can be attributed to the high microbiological quality variability, observed during the analysis, which is in agreement with other water reuse studies performed by other authors (McLain and Williams, 2012; Palese et al., 2009). Significant differences ($p<0.05$) were observed in the aerobic bacterial counts, between the secondary-treated wastewater and the other waters. Among the results obtained for the groundwater and disinfected reclaimed water samples, no significant differences ($p>0.05$) were found. This result highlights the importance of having an on-site disinfection process, to ensure the final quality of the irrigation water. It is important to note the criticalrole of storage in wastewater quality changes (Cirelli et al., 2008). Water quality

can degrade by the time it gets to the point of use (Jjemba et al., 2010). An effective storage step for secondary-treated wastewater combined with an on-site disinfection step using an efficiently and easy to manage technology could be an appropriate scheme of water reuse. However, the long term effects of tertiary effluents discharge should be investigated to quantify environmental and health impacts, as well as its effect in the crop yield (Al-Sa'ed, 2007; Pedrero et al., 2013).

The qPCR technique has the advantage, among others, of enabling the detection of microorganisms difficult to cultivate, such as *Bacteroides* spp. and *Helicobacter pylori*. Shannon et al. (2007) have indicated that even though biological analytical methods usually used at WWTP are effective in determining the efficiency of the treatment process, these methods do not take into account the presence of other pathogens which may be introduced to the wastewater system, nor do they consider the infectious dose of other pathogens which may be in much smaller number than the indicators.

As mentioned in Chapter 2 of this dissertation (section 2.2.2. Biological agents) *Bacteroides* detection can be a reliable and accurate method to estimate fecal concentrations in water samples (Layton et al., 2006). *Bacteroides* spp. only has been detected by qPCR in two secondary-treated wastewater samples, but these cells were not detected in v-qPCR. Therefore, the found results indicate that the secondary-treatment was efficient in inactivating them. During the secondary-treatment the oxygen levels can be high, and *Bacteroides,* which are anaerobic, could be unable to grow in an oxygenated environment. Ballesté and Blanch (2010) have done an interesting study to improve understanding of *Bacteroides* species survival in the environment. Their on-site experiments showed different survival patterns for the cultivable *Bacteroides* strains. For environmental *Bacteroides* spp. dissolved oxygen concentration in water was the variable that more affected the die-off.

Helicobacter pylori should be a pathogen to consider in the case of reuse of water for irrigation of vegetables, especially those that are consumed uncooked because they can cause peptic ulcers and gastric diseases and play an important role in gastric cancer (Kenneth and McColl, 2010). *Helicobacter pylori* were detected by qPCR, at least one time in the four types of monitored waters. The v-qPCR showed positive results of 4 log units for one secondary-treated wastewater sample and a 3 log units for one reclaimed water (+ photocatalysis) sample. Other reclaimed water (+ photocatalysis) sample was positive by v-qPCR, but when the triple approach was applied, the background signal of PMA treated dead cells was very similar to the signal from the PMA treated cells, so the viability of the cells was undetermined. As it has been previously mentioned in Chapter 6 of this dissertation, the usefulness of the viability PCR technique, when photocatalysis disinfection is used to kill cells requires to be further studied.

Microbiological quality of irrigated vegetables

The microbiological quality of vegetables irrigated with different water qualities was studied. Two washes with sterile PBS were performed before the tissue analysis was carried out, in order to have an idea of the surface contamination.

Escherichia coli was not detected in wash water from lettuce or string beans samples, but it was positive for the carrot water wash samples. A concentration of about 1 log unit per gram of analyzed vegetable was observed for groundwater and both disinfected reclaimed waters. The microbial load was 2 log units for secondary-treated wastewater. In the second washing counts were closer to the detection limit, 1 CFU/g. The positive detection of this microbial indicator in carrot water wash samples could be related to the issue that carrots are root crops, therefore the source of contamination could be the soil and not the irrigation water (Beuchat, 1996).

Escherichia coli was not detected by culture in any of the analyzed plant tissue samples and only one sample of lettuce irrigated with secondary-treated wastewater was positive by qPCR. The viability of the cells could not be confirmed, because v-qPCR was not performed with vegetable tissue samples, due to the high turbidity of the samples and its high inhibition levels. The contamination of these samples could be superficial or bacteria could be

internalized within the tissues. Sapers (2001) previously mentioned that some factors such as the attachment of bacteria to inaccessible sites or they incorporation into biofilms may limit the washing efficacy.

Enterococci, *Helycobacter pylori*, and *Bacteroides* spp. were not detected in vegetable tissue samples analyzed. On other hand, some positive results were found in wash water samples indicating that contamination could be superficial. Bechat (1996) highlighted soil, air, and animals as possible sources of microbial pathogens on fresh produce at the pre-harvest stage. Enterococci positive results were observed in the wash water samples from string beans. However, the found bacterial load was below 50 CFU/g from all cases. Two wash water samples from lettuce irrigated with secondary-treated wastewater were positive for *Helicobacter pylori* by qPCR, although based on the v-qPCR results these cells were not viable. One wash water sample from lettuce irrigated with secondary-treated wastewater resulted positive for *Bacteroides* spp., with an approximate concentration of 100 CFU/g, according to the results obtained by v-qPCR.

These results allow the visualization of the importance to analyze the presence of pathogens other than conventional, in order to obtain a more realistic idea of the sanitary quality of the samples.

Coliform bacterial counts were similar for the three vegetables studied. Surface contamination was reduced in approximately 1 log unit between the first washing and between the second washing. In general counts resulted to be a bit smaller for groundwater and a little higher for secondary-treated wastewater. Contamination of vegetable tissue was, in almost all cases, greater than the surface contamination, staying between 5 and 2 log units per gram of fresh plant analyzed.

In the case of heterotrophic bacteria, contamination could be only superficial because the bacterial load found for vegetable material was lower than that of surface contamination (wash water samples). Counts were between 6 and 5 log units.

Based on the results observed from the determination of microbiological quality, of both irrigation water and irrigated vegetables, it can be said that the secondary-treated wastewater used in this study presented a good microbial quality. Moreover, the use of some kind of *in situ* disinfection ensures that good quality water will be used for crop irrigation taking into account the water microbiological quality variation, both in the effluent and during the reclaimed water storage step. Adequate reclamation including disinfection is a core factor for successful and sustainable wastewater reuse (Al-Sa'ed, 2007).

The quality of the secondary-treated wastewater from the 90% of cases was in compliance the regulations established by the RD 1620/2007. On the other hand, the quality of reclaimed water with *in situ* disinfection process was in 100% of cases within the established by the Royal Decree. Previous studies have also demonstrated that, under suitable conditions, reclaimed water can be useful as an additional water resource for crop irrigation (Chen et al., 2013; Cirelli et al., 2012; Pollice et al., 2004).

It is important to notice the importance of vegetables washing, especially those eaten raw, as they have showed a reduction in the microbial load of about 1-1.5 log units, between the first and second washing.

Regarding the microorganism detection techniques used in this study, it is worth highlight the advantages of using molecular biology techniques, such as qPCR. The qPCR is a sensitive detection technique which provides fast and reliable results, and also facilitates the detection of fastidious organisms. In addition to all these advantages, the v-qPCR approach allows to distinguish between live and dead cells (Fittipaldi et al., 2011). Although further research is needed to optimize this technique (Fittipaldi et al., 2012), Varma et al. (2009) have concluded that PMA-qPCR method can be used to predict the microorganisms' elimination efficiency from usual wastewater treatment process. Therefore, it can be a useful microbial monitoring tool.

It should be noted that while the qPCR has ample advantages, a major drawback are the inhibition problems suffered by the technique, which may lead to false negative results. Opel

et al. (2010) have found that a variety of inhibition mechanisms can occur during the PCR process, depending on the type of the co-extracted inhibitor. However, this disadvantage can be solved using both internal controls in the qPCR reaction and serial dilutions, which dilute inhibitors present in the samples. Also, it is important to play special attention to the DNA extraction and purification step, as well as the use of good quality reagents.

Some problems of inhibition were observed in our work, when the vegetable samples were analyzed. Wash water samples from lettuce and string beans showed a low inhibition profile (requiring a dilution of 1/10), and even in some cases any inhibition was seen. However, for carrot wash water, inhibition was very strong (requiring dilution of 1/1000), probably because the samples had more soil particles compared to other plants, and therefore higher content of humic acids, compounds which are strongly PCR inhibitors. For vegetable tissues the inhibition was higher, requiring sample dilutions of 1/100 and 1/1000, thus increasing the limit of detection of the technique and detracting its usefulness for the vegetable microbiological quality monitoring, at least in terms of fecal microorganisms which are easy to cultivate. Chlorophylls, humic substances and polysaccharides originating from vegetables have shown to be PCR inhibitors (Schrader et al., 2012; Wilson, 1997). Thus, qPCR methods are highly dependent on the DNA extraction and purification techniques. However, the develop of novel and optimized DNA extraction and/or purification strategies has been continued during the years, especially for complex samples such as clinical (Richardson et al., 2012), food (Rodríguez-Lázaro et al., 2013), and environmental (Rodriguez et al., 2012) samples.

Herein, the viability qPCR method was not applied to vegetable material samples due to the high presence of biomass in them and their turbidity. Previous studies have demonstrated that a high amount of suspended solids could interfere with the ability of PMA to link DNA from damaged cells (Bae and Wuertz, 2009; Varma et al., 2009; Wagner et al., 2008). However, it is important to notice that some studies about the suitable use of v-PCR technique for live cells of *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 detection from artificially inoculated vegetables, have been recently published (Elizaquível et al., 2012a; Elizaquível et al., 2012b; Liang et al., 2011). Also, the detection of *Candidatus liberibacter* asiaticus from citrus samples was performed using EMA-qPCR (Trivedi et al., 2009).

7.5. Conclusion

In conclusion, the analyzed conditions present a favorable scenario for the use of reclaimed water, as long as it complies with a minimum quality parameters, established by the RD1620/2007.

This study confirms that, under the conditions tested, good quality reclaimed water can be an additional water resource for irrigation. The study also highlights the security involved in the microbial quality using an *in situ* disinfection process, as reclaimed water use is concerned. The importance of the one or two washing steps has also been demonstrated, especially for raw consumption products.

Health hazards are one of the main constraints for non-potable reclaimed water use, so controlling and monitoring of water are effective targets for contamination prevention. For that, precise and fast control tools such as qPCR, are needed. They are valuable because enables a faster and reliable determination of specific microorganisms. The v-qPCR methods used in this study to monitor the microbiological quality from the irrigation water were effective in the detection of pathogenic and non-pathogenic microorganisms, in the four different types of studied water. These techniques could be useful tools for the implementation of quality control programs, the Hazard Analysis Critical Control Point (HACCP) and good manufacturing practices. Also, they could play an important role in the monitoring of disinfection treatments for WWTP and industries. Although, it is important to

clear that their usefulness will depend on the used disinfection method damages the cell membrane or not.

The obtained results in this study could indicate the microbial risk associated with a simple best-case scenario. Further studies associated with a simple worst-case scenario will be of significant value, in managing reclaimed water reuse schemes. More studies should be performed with the ultimate goal of increasing confidence in the reuse of treated wastewater.

7.6. References

Agusti, G., Codony, F., Fittipaldi, M., Adrados, B., Morató, J. 2010. Viability determination of *Helicobacter pylori* using propidium monoazide quantitative PCR. Helicobacter, 15, pp. 473–476.

Alonso, M.C., Dionisio, L.P.C., Bosch, A., Pereira de Moura, B.S., Garcia-Rosado, E., Borrego, J.J. 2006. Microbiological quality of reclaimed water used for golf courses' irrigation. Water Sci. Technol., 54, pp. 109-117.

Al-Sa'ed, R. 2007. Pathogens assessment in reclaimed effluent used for industrial crop irrigation. Int. J. Environ. Res. Public Health, 4, pp. 68-75.

Álvarez, P.M., Beltrán, F.J., Jaramillo, J., Pocostales, P., Márquez, G. 2011. Comparison of various advanced treatment methods for municipal wastewater reclamation. WASET, 55, pp. 653-654.

Asano, T. 1998. Wastewater reclamation and Reuse, Water Quality Management (ed.) Library, 10 CRC Press, Florida, USA.

Bae, S., Wuertz, S. 2009. Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with propidium monoazide. Appl. Environ. Microbiol., 75, pp. 2940-2944.

Ballesté, E., Blanch, A.R. 2010. Persistence of *Bacteroides* species populations in a river as measured by molecular and culture techniques. Appl. Environ. Microbiol., 76, pp. 7608-7616.

Beuchat, L. R. 1996. Pathogenic microorganisms associated with fresh produce. J. Food Prot., 59, pp. 204-216.

Chen, W., Lu, S., Jiao, W., Wang, M., Chang, A.C. 2013. Reclaimed water: a safe irrigation water source?. Environ. Development, 8, pp. 74-83.

Cheng, Y.W., Chan, R.C.Y., Wong, P.K. 2007. Disinfection of *Legionella pneumophila* by photocatalytic oxidation. Water Res., 41, pp. 842-852.

Cirelli, G.L., Consoli, S., Di Grande, V. 2008. Long-term storage of reclaimed water: the case studies in Sicily (Italy). Desalination, 218, pp. 62-73.

Cirelli, G.L., Consoli, S., Licciardello, F., Aiello, R., Giuffrida, F., Leonardi, C. 2012. Treated municipal wastewater reuse in vegetable production. Agr. Water Manage., 104, pp. 163-170.

Clark, C.S. 1987. Potential and actual biological related health risks of wastewater industry employment. J. Water Poll. Control Fed., 59, pp. 999-1008.

Crittenden J.C., Trussell R.R., Hand D.W., Howe K.J., Tchobanoglous G. 2005. Water treatment: principles and design. Second Edition. Wiley. New Jersey, US.

Directive 91/676/EEC. European Commission. 1991. Protection of waters against pollution caused by nitrates from agricultural sources. Office for Official Publications of the European Communities.

Directive 91/271/ECC. European Commission. 1991. Urban wastewater treatment. Official Journal of the European Communities, 135 (30.5).

Directive 98/83/EC. 1998. The quality of water intended for human consumption. Official Journal of the European Communities, 330, pp. 5-12.

Elizaquível, P., Sánchez, G., Selma, M.V., Aznar, R. 2012a. Application of propidium monoazide-qPCR to evaluate the ultrasonic inactivation of *Escherichia coli* O157:H7 in fresh-cut vegetable wash water. Food Microbiol., 30, pp. 316-320.

Elizaquível, P., Sánchez, G., Aznar, R. 2012b. Quantitative detection of viable foodborne *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* in fresh-cut vegetables combinig propidium monoazide and real-time PCR. Food Control, 25, pp. 704-708.

EPSAR (Entidad Pública de Saneamiento de Aguas Residuales de la Comunidad Valenciana). 2012. Reutilización de aguas depuradas en la comunidad valenciana. Oral presentation. IX Congreso Internacionala AEDyR. November, 13-15. Madrid, Spain.

European Food Safety Authority. 2010. Statement on possible public health risks for infants and young children from the presence of nitrates in leafy vegetables. EFSA Journal, 8 (12) 1935, pp. 1-42.

E.Z.N.A.® Tissue DNA Kit Handbok. 2012. Cultured cells spin protocol, pp. 12-15. Available in: http://www.omegabiotek.com.

FAO. 2007. Coping with water scarcity- Challenge of the twenty-first Century. In: de Miguel, A., Martínez-Hernández, V., Leal, M., González-Naranjo, V., de Bustamante, I., Lillo, J., Martin, I., Salas, J.J., Palacios-Díaz, M.P. 2013. Short-term effects of reclaimed water irrigation: *Jatropha curcas* L. cultivation. Ecol. Eng., 50, pp. 44-51.

Fittipaldi, M., Codony, F., Adrados, B., Camper, A. K., Morató, J. 2011. Viable real-time PCR in environmental samples: can all data be interpreted directly?. Microb. Ecol., 61, pp. 7-12.

Fittipaldi, M., Nocker, A., Codony, F. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J. Microbiol. Methods, 91, pp. 276-289.

Garbisu, C., Blanco, A., Alkorta, I., Llama, M. J., Sierra, J. L. 1999. Biotecnología con cianobacterias. Investigación y Ciencia, pp. 64-71.

Gómez, M., de la Rua, A., Garralón, G., Plaza, F., Hontoria, E., Gómez, M.A. 2006. Urban wastewater disinfection by filtration technologies. Desalination, 190, pp. 16-28.

Hoefel, D., Monis, P.T., Grooby, W.L., Andrews, S., Saint, C.P. 2005. Culture-independent techniques for rapid detection of bacteria associated with loss of chloramine residual in a drinking water system. Appl. Environ. Microbiol., 71, pp. 6479-6488.

Huang, S.W., Hsu, B.M., Ma, P.H., Chien, K.T. 2009. *Legionella* prevalence in wastewater treatment plants of Taiwan. Water Sci. Technol., 60, pp. 1303-1310.

ISO 6222. 1999. Water quality -Enumeration of culturable micro-organisms - Colony count by inoculation in a nutrient agar culture medium.

Jaworska, G. 2005. Content of nitrates, nitrites, and oxalates in New Zealand spinach. Food Chemistry, 89, pp. 235-242.

Jiménez, B., Asano, T. 2008. Water reuse. An international survey of current practice issues and needs, 1 st edn. IWA, London.

Jjemba, P.K., Weinrich, L., Cheng, W., Giraldo, E., LeChavallier, M.W. 2010. Guidance document on the microbiological quality and biostability of reclaimed water following storage and distribution. WateReuse Foundation, Virginia, US.

Kellis, M., Kalavrouziotis, I.K., Gikas, P. 2013. Review of wastewater reuse in the Mediterranean countries, focusing on regulations and policies for municipal and industrial applications. Global Nest J., 15, pp. 333-350.

Kobayashi, D., Eishi, Y., Ohkusa, T., Ishige, I., Suzuki, T., Minami, J., Yamada, T., Takizawa, T. 2002. Gastric mucosal density of *Helicobacter pylori* estimated by real-time PCR compared with results of urea breath test and histological grading. J. Med. Microbiol., 51, pp. 305–311.

Layton, A., McKay, L., Williams, D., Garret, V., Gentry, R., Sayler, G. 2006. Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for eEstimation of total, human, and bovine fecal pollution in water. Appl. Environ. Microbiol., 72, pp. 4214-4224.

Lazarova, V., Bahri, A. 2005. Water reuse for irrigation. Agriculture, landscape, and turf grass. CRC Press, Florida, US.

Lee, J., Kim, E., Roh, B., Eom, S., Zoh, K. 2013. Occurrence of disinfection by-products in tap water distribution systems and their associated health risk. Environ. Monit. Assess., 185, pp. 7675-7691.

Levantesi, C., La Mantia, R., Masciopinto, C., Böckelmann, U., Ayuso-Gabella, M.N., Salgot, M., Tandoi, V., Van Houtte, E., Wintgens, T., Grohmann, E. 2010. Quantification of pathogenic microorganisms and microbial indicators in three wastewater reclamation and managed aquifer recharge facilities in Europe. Sci. Total Environ., 408, pp. 4923-4930.

Liang, N., Dong, J., Luo, L., Li, Y. 2011. Detection of viable *Salmonella* in lettuce by propidium monoazide real-time PCR. J. Food Sci., 76, pp. M234-M237.

Lydakis-Simantirisa, N., Rigaa, D., Katsivelaa, E., Mantzavinosb, D., Xekoukoulotakisa, N.P. 2010. Disinfection of spring water and secondary treated municipal wastewater by TiO2 photocatalysis. Desalination, 250, pp. 351-355.

McCullagh, W.L., Robertson, J.M.C., Bahnemann, D.W., Robertson, P.K.J. 2007. The application of TiO2 photocatalysis for disinfection of water contaminated with pathogenic microorganisms: a review. Res. Chem. Intermediat., 33, pp. 359-375.

McLain, J.E.T., Williams, C.F. 2012. Assessing environmental impacts of treated wastewater through monitoring of fecal indicator bacteria and salinity in irrigated soils. Environ. Monit. Assess., 184, pp. 1559-1572.

Moghadam, A.K., Dore, M. 2012. Cost and efficacy of water disinfection practices: evidence from Canada. Rev. Econ. Anal., 4, pp. 209-223.

Mujeriego, R. 1990. Manual Práctico de Riego con Agua Residual Municipal Regenerada. Ediciones de la Universitat Politècnica de Catalunya, Barcelona.

Muñoz, I., Tomàs, N., Mas, J., García-Reyes, J.F., Molina-Díaz, A., Fernández-Alba, A.R. 2010. Potential chemical and microbiological risks on human health from urban wastewater reuse in agricultura. Case study of wastewater effluents in Spain. J. Environ. Sci. Health B, 45, pp. 300-309.

Nakano, R., Ishiguro, H., Yao, Y., Kajioka, J., Fujishima, A., Sunada, K., Minoshima, M., Hashimoto, K., Kubota, Y. 2012. Photocatalytic inactivation of influenza virus by titanium dioxide thin film. Photochem. Photobiol. Sci., 11, pp. 1293-1298.

Omar, K.B., Barnard, T.G. 2010. The occurrence of pathogenic *Escherichia coli* in South African wastewater treatment plants as detected by multiplex PCR. Water SA, 36, pp. 172-176.

Opel, K.L., Chung, D., McCord, B.R. 2010. A Study of PCR inhibition mechanisms using real time PCR. J. Forensic Sci., 55, pp. 25-33.

Palese, A.M., Pasquale, V., Celano, G., Figliuolo, G., Masi, S., Xiloyannis, C. 2009. Irrigation of olive groves in Southern Italy with treated municipal wastewater: effects on microbiological quality of soil and fruits. Agric. Ecosyst. Environ., 129, pp. 43-51.

Pedrero, F., Kalavrouziotis, I., Alarcón, J.J. Koukoulakis, P., Asano, T. 2010. Use of treated municipal wastewater in irrigated agriculture-Review of some practices in Spain and Greece. Agr. Water Manage., 97, pp. 1233-1241.

Pedrero, F. 2010. Sustainable irrigation management with reclaimed water. Doctoral dissertation. Consejo Superior de Investigaciones Científicas (CSIC). Murcia, Spain.

Pedrero, F., Mounzer, O., Alarcón, J.J., Bayona, J.M., Nicolás, E. 2013. The viability of irrigating mandarin trees with saline reclaimed water in a semi-arid Mediterranean region: a preliminary assessment. Irrig. Sci., 31, pp. 759-768.

Pérez, L.M., Codony, F., López Leyton, D., Fittipaldi, M., Adrados, B., Morató J. 2010. Quantification of *Helicobacter pylori* levels in soil samples from public playgrounds in Spain. J. Zhejiang Univ. Sci. B, 11, pp. 27–29.

Pollice, A., Lopez, A., Laera, G., Rubino, P., Lonigro, A. 2004. Tertiary filtered municipal wastewater as alternative water source in agriculture: a field investigation in Southern Italy. Sci. Total Environ., 324, pp. 201-210.

Pourcher, A., Picard-Bonnaud, F., Ferré, V., Gosinska, A., Stan, V., Moguedet, G. 2007. Survival of faecal indicators and enteroviruses in soil after land-spreading of municipal sewage sludge. Appl. Soil Ecol., 35, pp. 473-479.

Richardson, L.J., Kaestli, M., Mayo, M., Bowers, J.R., Tuanyok, A., Schupp, J., Engelthaler, D., Wagner, D.M., Keimb, P.S., Curriea, B.J. 2012. Towards a rapid molecular diagnostic for melioidosis: Comparison of DNA extraction methods from clinical specimens. J. Microbiol. Methods, 88, pp. 179-181.

Robertson, J.M.C., Lawton, L.A., Robertson, P.K.J. 2005. A comparison of the effectiveness of TiO_2 photocatalysis and UVA photolysis for the destruction of three pathogenic microorganisms. J.Photochem. Photobiol. Sci. A: chemistry, 175, pp. 51-56.

Rodríguez, R.A., Thie, L., Gibbons, C.D., Sobsey, M.D. 2012. Reducing the effects of environmental inhibition in quantitative real-time PCR detection of adenovirus and norovirus in recreational seawaters. J. Virol. Methods, 181, pp. 43-50.

Rodríguez-Lázaro, D., Cook, N., Hernández, M. 2013. Real-time PCR in food science: PCR diagnostics. Curr. Issues Mol. Biol., 15, pp. 39-44.

Royal Decree-Law 1620/2007 of treated wastewater reuse. 2007. Official Bulletin of State, BOE, 294.

Royal Decree-Law 1138/1990 which approved the technical-sanitary regulations for the supplying and water quality for public consumption. Official Bulletin of State, BOE, 226.

Salgot, M., Huertas, E., Weber, S., Dott, W., Hollender, J. 2006. Wastewater reuse and risk: definition of key objectives. Desalination, 187, pp. 29-40.

Sapers, G.M. 2001. Efficacy of washing and sanitizing methods for disinfection of fresh fruit and vegetable products. Food Technol. Biotechnol., 39, pp. 305-311.

Schrader, C., Shielke, A., Ellerbroek, L., Johne, R. 2012. PCR inhibitors–occurrence, properties and removal. J. Appl. Microbiol., 113, pp. 1014-1026.

Shannon, K.E., Lee, D.Y., Trevors, J.T., Beaudette, L.A. 2007. Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. Sci. Total Environ., 382, pp. 121-129.

Solsona, F., Mendéz, J.P. 2002. Desinfección de agua. Centro Panamericano de Ingeniería Sanitaria y Ciencias del ambiente (CEPIS). Lima, Perú. http://www.bvsde.paho.org/bvsacg/fulltext/desinfeccion/desinfeccion.html

Stevens, D.P., Smolenaars, S., Kelly, J. 2008. Irrigation of amenity horticulture with recycled water. Arris Pty. Ltd. Melbourne, Australia, pp. 49.

Sunnotel, O., Verdoold, R., Dunlop, P.S.M., Snelling, W.J., Lowery, C.J., Dooley ,J.S.G., Moore, J.E., Byrne, J.A. 2010. Photocatalytic inactivation of *Cryptosporidium parvum* on nanostructured titanium dioxide films. J. Water Health, 8, pp. 83-91.

Tillman, D. 1999. Global environmental impacts of agricultural expansion: the need for sustainable and efficient practices. Proc. National Academy of Sci. USA. 96, pp. 5995-6000.

Trivedi, P., Sagaram, U.S., Kim, J.S., Briansky, R.H, Rogers, M.E., Stelinski, L.L., Oswalt, C., Wang, N. 2009. Quantification of viable *Candidatus Liberibacter asiaticus* in hosts using quantitative PCR with the aid of ethidium monoazide (EMA). Eur. J. Plant Pathol., 124, pp. 553-563.

UNE-EN 872:2006. Water quality. Determination of suspended solids. Method by filtration through glass fibre filters. AENOR, AEN/CTN 77-Environment Committee.

Varma, M., Field, R., Stinson, M., Rukovets, B., Wymer, L., Haugland, R. 2009. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. Water Res., 43, pp. 4790–4801.

Wagner, A.O., Malin, C., Knapp, B.A., Illmer, P. 2008. Removal of free extracellular DNA from environmental samples by ethidium monoazide and propidium monoazide. Appl. Environ. Microbiol., 74, pp. 2537–2539.

Wang, J., Liu, X., Ng, T.W., Xiao, J., Chow, A.T., Wong, P.K. 2013. Disinfection byproduct formation from chlorination of pure bacterial cells and pipeline biofilms. Water Res., 47, pp. 2701-2709.

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol., 63, pp. 3741–3751.

Yates, M.V. 1997. Monitoring concerns and procedures for human health effects. In: Wastewater Reuse for Golf Course Irrigation, US Golf Association (ed.). Lewis Publisher, Florida, US, pp. 143-171.

Chapter 8

General conclusions and recommendations

*"It would be an attractive idea to describe our advances in knowledge as a series of logically planned and orderly steps from darkness and ignorance to light and understanding. Unfortunely, in our experience, progress rarely occurs like this. Research workers in more than one field have linkened their experience to walking across a darkened room, constantly bumping into various objects. Only later, when eyes adjust to the gloom, can they look back and see exactly how the furniture is arranged. But that is only part of the story. When they switch on the light they discover another door and another darkened room. And the whole process is repetead."(*David Tyrrel, 2002).[1]

[1] Tyrrell, D., Fielder, M. 2002. Cold wars: the fight against the common cold. Oxford University Press, Great Britain. Preface, pp. VIII.

8.1. General conclusions

Wastewater generation is an inevitable component of human activities. However, it can be reduced if water is more effectively used. Moreover, wastewater can be regenerated and reused providing an opportunity for water conservation and reduction of effluent discharges into receiving waters. Thus, reclaimed water is an integral part of the water cycle and contributes to guarantee the water availability in terms of both quantity and quality.

As it was mentioned in the introduction, this dissertation aimed to contribute and increase the available knowledge on microbiological quality of reclaimed water and its monitoring, and on the study of potential sanitary risk associated to agricultural and industrial reclaimed water use practices, in order to contribute to the sustainable and safe-use of reclaimed water. To achieve these main objectives, work was performed along two main interlinked lines of research: (1) the study of qPCR methods as water microbiological quality monitoring tools, and (2) the study of microbiological colonization associated to reclaimed water use at pilot scale practices in the Catalonian region using culture and qPCR techniques.

Initially, an approximation to molecular methods was achieved by optimizing a qPCR method to detect *Legionella pneumophila* in water samples, especially cooling water samples. Most of the work was performed working with *Legionella* detection because it is an opportunistic pathogen of significant public concern and its monitoring is crucial for risk assessment and for providing information to identify control strategies. The optimized method resulted to be a powerful screening tool for monitoring *Legionella pneumophila* in hot water and cooling water samples, allowing for fast and reliable results. It could play a key role in the context of a health risk management program, and also during an outbreak investigation. In this a study, a lack of correlation was observed between both of used methods (culture and qPCR) which highlight the need to develop an acceptable standardized method to detect *Legionella* in a timely fashion with a high degree of sensitivity and specificity. In this sense, qPCR showed to be a powerful screening tool and could play an important role, especially if viability information can be added.

The need to add viability information to qPCR techniques was identified in order to improve them and their implementation in routine monitoring applications. The development of viability qPCR method means a step forward in the field of molecular techniques because it makes use of the speed of molecular detection methods while, at the same time, provides viability information. For this reason in-depth work was performed with viability qPCR aiming at improving this technique, with special attention to its use for environmental samples. The viability qPCR technique using ethidium monoazide (EMA) or propidium monoazide (PMA) was validated using pure cultured and environmental samples. The addition of a pre-treatment step to the sample analysis to inhibit the amplification of DNA from membrane-damaged cells has been used in combination with qPCR to detect live *Legionella pneumophila*, infective bacteriophage T4 (Fittipaldi et al., 2010), viable *Acanthamoeba castellani* trophozoites, and cysts (Fittipaldi et al., 2011), *Helicobacter pylori*, *Bacteroides* spp. and *Escherichia coli* through experimental work performed during the course of this dissertation. Thus, a broad experience in this research area was achieved. Chapters 5 and 6 depicted some studies of application of PMA pre-treatment to detect and quantify live microorganisms using molecular methods. In Chapter 5 the ability of viability qPCR to quantify viable *Legionella pneumophila* in the presence of dead cells has been analyzed. Some limitations of this technique to quantify properly viable cells in the presence of high levels of dead cells were identified, since the qPCR signal was not suppressed entirely and false-positive results were obtained. Under the study conditions false estimates of live cells were obtained when the concentration of dead cells was larger, in a factor approximately to 10^2, than the concentration of live cells. Thus, the herein used viable qPCR protocol, by itself without additional improvements, was not suitable for the correct quantification of *Legionella pneumophila* in environmental samples with a high number of dead cells, and/or high contaminated samples exposed to disinfection treatments. However, it is important to note that, in these cases, the additional analysis of a non-concentrated sample by v-qPCR, as

it is currently used in *Legionella* culture analysis from environmental samples, may be useful. Additionally, plate counts could be performed to discard false-positive results.

In part the v-qPCR observed limitations can be minimized by choosing experimental variables and conditions adequate for a particular sample. In this regard, a review summarizing current knowledge and presenting aspects which are important when designing experiments employing viability dyes was published as result of the work performed through this dissertation (Fittipaldi et al., 2012). It is worth of mention that the choice of an adequate light source to perform the photoactivation step could be important in regard of the technique efficiency. Photoactivation step using halogen lamps showed to be problematic. LED light sources can be considered beneficial to prevent unnecessary heating, to minimize DNA damage by wavelengths others than the one required for dye activation, and to avoid unnecessary optimization of light exposure time and testing of the suitability of the light source. For this reason a LED photoactivation system was used in the subsequent studies.

Considering the possible technique limitations and taking into account that protocol optimization for each sample and each microorganism is not always easy to achieve, a strategy involving three independent qPCR reactions on identical sample aliquots to minimize the influence of false-positive and false-negative results was proposed and studied in Chapter 6. This strategy was based on the combination of one regular qPCR reaction, one viable qPCR reaction, and one viable qPCR reaction on an aliquot subjected to lethal conditions inflicting membrane damage, with the intent to provide more objective data regarding the number of live microbes by comparison and subtraction of results. At least for the analyzed samples, the used microbial targets, and cell damage caused by disinfection, the feasibility of the approach for a realistic estimate of the number of live cells has been validated. In absence of robust and reliable procedures, and keeping in mind that in microbiology it is very difficult to have accurate results on viability assessment due to the heterogeneous nature of microbial life, the concept as outlined in this work is interesting as a future research direction and it also offers a better understanding of microbial dynamics in complex samples.

The optimized qPCR and v-qPCR methods were employed to study the use of reclaimed water in cooling towers pilot systems and also, in a pilot study encompassed to ascertain the safety of irrigating vegetables crops with reclaimed water. In both studies similar conclusions were achieved. Under the studied conditions, the use of secondary-treated wastewater had the highest levels of bacterial load. While, reclaimed water with some kind of disinfection treatment behaved equivalent to untreated well water with regard to the risk of *Legionella* colonization and biological growth in general. Thus, the use of disinfected reclaimed water does not entail greater pollution when compared to well water. This fact emphasizes the importance of disinfection treatment *prior* to use for the case of reclaimed water. Therefore, reclaimed water can be useful as an additional source of water under suitable and controlled conditions.

Application of on-site disinfection at the end-use point or/and the storage step is highly recommended, especially considering the great variability in microbial quality that different reclaimed water batches can have and the loss of quality that water can suffer during distribution and storage steps. Moreover, if end-point disinfection is used, good quality secondary-treated wastewater is capable of being used, as it was demonstrated in this work.The importance of the application of one or two washing steps when vegetables are irrigated with reclaimed water has also been demonstrated. This is especially important for products that will be consumed raw.

In conclusion, the analyzed conditions present a favorable scenario for the reuse of reclaimed water, as long as it complies with a minimum quality parameters established in the RD1620/2007.

In these pilot studies the PMA-qPCR technique showed, in general, the same microbial colonization dynamic and similar pollution levels than culture, therefore the same but faster conclusions can be achieved when this technique is used. However, some DNA amplification inhibition problems were observed when vegetables samples were analyzed. Thus, technical and procedures improvements are required and should be addressed in future studies.

Regarding *Legionella* detection, it is important to note that a large number of results were indeterminable by culture standard method due to the presence of high levels of other microbiota which hampers *Legionella* isolation. Therefore, qPCR resulted to be a useful monitoring tool that can be used accompanied with culture methods. Quantitative PCR methods are valuable because they enable a faster and reliable determination of specific microorganisms. Moreover, overestimation of the pathogen concentration can be better that underestimation or no estimation at all. Since the public harm caused by the failure (false-negative results or underestimated concentrations) to detect and treat systems or products containing pathogens may be ultimately considered greater than the cost of anticipative treatments.

The work done for this dissertation contributes to reduce persistent uncertainty in relation to the potential adverse effects that may encompass the use of reclaimed water on human health, with the ultimate goal of increasing confidence in reuse practices and public acceptance. Health hazards are one of the main constraints for non-potable reclaimed water use, so the control and monitoring of water are effective targets for contamination prevention. Improved detection of microbial pathogens in reclaimed water will be essential to help optimizing all steps in treatment and use of reclaimed wastewater in agricultural and industrial uses. Quantitative PCR has the potential to be one of the quickest and most useful methods available for microbial pathogen detection. Amplification is typically an essential requirement when using molecular technology for the detection of pathogens which can be present in very low numbers. Treatment with viability dyes profits from the fact that it is easy to perform, compatible with existing technology and does not significantly increase the time to results. Despite the need for a more efficient exclusion of dead cells, sample treatment with viability dyes with subsequent amplification of DNA can be seen as highly valuable for a wide range of applications.

Viable qPCR techniques let perform a rapid screening of water quality while obtaining timely and reliable data, thus allowing for quick response time for decision making. This technique is also a powerful monitoring tool that allows following disinfection process and assessing changes in water quality. Therefore it could be an useful tool for the implementation of microbial quality control programs. Additionally, the herein developed and applied triple qPCR approach might help to reduce overestimation of bacterial viability in complex matrices like wastewater samples, particularly when protocol optimization will be difficult to be performed.

As established Dr. Lucas Van Vuuren *"water should be judged by its quality; not its history"* (Howe and Mitchell, 2012) and viability qPCR is a good tool to achieve this.

8.2. Recommendations

8.2.1. Reclaimed water

During this dissertation work, pilot studies were performed to ascertain the safety of reclaimed water application for industrial and agricultural uses. Regarding the cooling tower pilot study, stronger conclusions require more experiments, carried out in pilot plants simulating real operation conditions of cooling towers, with larger number of samples and multiple experimental replicates. Regarding the vegetable irrigation pilot study, the obtained results could indicate the microbial risk associated with a simple best-case scenario. Further studies associated with a simple worst-case scenario would be of significant value in managing reclaimed water reuse schemes. The prevalence of *Helycobacter pylori* in vegetables irrigated with reclaimed water should be further studied in order to incorporate it as a microbiological parameter in guidelines. Moreover, the long-term effect of water reuse in the environment, in the health aspects related to chemical pollutants and in the crop production should be taken into account.

The importance played by biofilms as the most important role in the microbial dynamics in installations, such as cooling towers or water recirculating systems, has been identified. Thus, further work is necessary to devise disinfection processes to control or to eliminate attached cells.

In order to draw more detailed conclusions on reclaimed water use, a cost analysis of implementing and using reclaimed water should be conducted.

8.2.2. Viability qPCR techniques

Although the development of the viable qPCR technique is an important step forward in the quantification of live cells by DNA detection based methods, for the successful application of this method several factors that can influence the outcome of the resulting data have to be considered. Even though significant progress has been made in the last years to find better v-qPCR protocols that selectively exclude dead cell DNA amplification, many questions about how efficiently the dyes bind and the impact of binding on DNA structure and function have still not been answered. Further studies are necessary to increase the method's capacity to suppress amplification signal from dead cells. In this sense, experimental evidence is necessary to have a better understanding of the influence of DNA sequence, DNA repair process, and the selectivity or preferential binding of the dyes for the different cell components in the v-qPCR technique efficiency.

More experimental information is required regarding the use of v-qPCR methods to detect live cells in biofilm and vegetables working in real conditions. Therefore, further research work is necessary in this area.

As the dyes have a limited capacity to exclude dead cell signals, the use of the triple v-qPCR approach developed in this work might help to identify the dead cell exclusion limit and ultimately improve the accuracy of live cell prediction. Moreover, environmental samples, due to the nature of their matrices, could require high optimization procedures in order to get a suitable v-qPCR protocol. In some specific cases, where that will be difficult, the triple approach could be also a good alternative to be applied. Thus, further studies in this research direction are recommended.

The principle of live-dead distinction in v-PCR is based on membrane integrity. Despite the common use of this conservative indicator for viability, it is important to understand its limitations. EMA or PMA like other membrane integrity stains do not measure "life" or "death", but assess a particular location-specific damage to cells (Hammes et al., 2011). Cell death can therefore only be diagnosed by v-qPCR if it is reflected in membrane damage. The diagnostic method cannot be applied to cells subjected to biocidal treatments that do not or only insufficiently inflict membrane damage in the relevant dose range with UV being a classical example. On the other hand, the treatments that affect membrane integrity vary in the degree of damage that is inflicted before cell death occurs. In regard to photocatalysis, the results obtained in the studies depicted in the Chapter 6 could indicate that the v-qPCR technique is not suitable to enumerate viable cells in samples treated with this method and further research is necessary.

8.3. References

Fittipaldi, M., Rodriguez, N. J., Codony, F., Adrados, B., Peñuela, G., Morató, J. 2010. Discrimination of infectious bacteriophage T4 virus by propidium monoazide real-time PCR. J. Virol. Methods, 168, pp. 228-232.

Fittipaldi, M., Pino Rodriguez, N.J., Adrados, B., Agustí, G., Peñuela, G., Morató, J. 2011. Discrimination of viable *Acanthamoeba castellani* trophozoites and cysts by propidium monoazide real-time polymerase chain reaction. J. Eukaryot. Microbiol., 58, pp. 359-364.

Fittipaldi, M., Nocker, A., Codony, F. 2012. Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. J. Microbiol. Methods, 91, pp. 276-289.

Hammes, F., Berney, M., Egli, T. 2011. Cultivation-independent assessment of bacterial viability. Adv. Biochem. Engin. Biotechnol., 124, pp. 123-150.

Howe, C., Mitchell, C. 2012. Water sensitive cities. IWA Publishing, London, UK, pp. 113