Universitat Autònoma de Barcelona

Integrated devices for the concentration and detection of waterborne bacteria

Josune Jimenez Ezenarro

Universitat Autònoma de Barcelona

··· Tesis doctoral ···

Integrated devices for the concentration and detection of waterborne bacteria

Departamento de Genética y Microbiología

Universitat Autònoma de Barcelona

··· Doctorado en Microbiología ···

··· Doctorado Industrial ···

Josune Jimenez Ezenarro

(2020)

Memoria presentada por Josune Jimenez Ezenarro para optar al grado de doctor en Microbiología por la Universidad Autónoma de Barcelona

Visto bueno de los directores de tesis:

Dr. Naroa Uria Moltó Dr. Francesc Xavier Muñoz-Pascual Dr. Jordi Mas Gordi

Tutor: Dr. Jordi Mas Gordi

DOCTORATS INDUSTRIALS Waterologies CNM CENTRO NACIONAL DE MICROELECTRONICA IMB

The thesis submitted by Josune Jimenez Ezenarro, graduate student in Biotechnology and Master degree in Advanced Biotechnology, entitled **Integrated devices for the concentration and detection of waterborne bacteria** was carried out at Universitat Autònoma de Barcelona (UAB) under the supervision of Dr. Naroa Uria Moltó, Post-Doc Researcher (IMB-CNM), and Dr. Fracesc Xavier Muñox Pascual (IMB-CNM), Senior Researcher, and Dr. Jordi Mas Gordi, Professor (UAB). The thesis satisfies the requirements for the title of Ph.D. in Microbiology.

This work has been performed within the program of Doctorats Industrials doctorates of the Generalitat of Catalunya with the company Waterologies S.L.

With the approval of:

Dr. Naroa UriaDr. Dr.Francesc Xavier Muñoz-Pascual Dr. Jordi Mas Gordi

Co-directors

Dr. Jordi Mas Gordi Josune Jimenez Ezenarro

Tutor **Author**

This Industrial PhD Project has been funded by the Departament d´Empresa I Coneixement of Generalitat de Catalunya (projects DI-018_2015 and DI-051_2017) and Waterologies S.L. The authors also acknowledge financial support from the Spanish Ministry of Economy and Competitiveness (SMARTECOPONICS project PCIN-2017-031) and Ministry of Science, Innovation and University [BACSYS projects CTQ2014-54553-C3-1-R and C3-2R] and [RTI2018-101974-B-C22].

Nire gurasoei, aiton-amonei eta Aitorri,

baita osaba, Peio, Miren eta Gorkari.

ABBREVIATIONS AND UNITS

Abbreviations:

Ab .....Antibody

ABCRS.....Automated bacterial concentration and recovery system

Abs .....Absorbance

AbsN .....Normalized absorbance

Ag/AgCl .....Silver/silver chloride

AMP .....Antimicrobial peptide

BCYE .....Buffered charcoal yeast extract

BSA .....Bovine serum albumin

CEIA .....Magnetic immunocapture with an enzyme-immunoassay

CFU .....Colony forming units

CV.....Cyclic voltammetry

DEP .....Dielectrophoresis

Chl-a.....Chlorophyll-a

DNA .....Deoxyribonucleic acid

EC .....Electrocoagulation

E. coli.....*Escherichia coli*

EHEC.....Enterohaemorrhagic *Escherichia coli*

EIEC .....Enteroinvasive *Escherichia coli*

ELISA.....Enzyme-linked immunosorbent assay

ELIFA.....Enzyme-linked Immunofiltration assay

EOF .....Electroosmotic flow

EPA.....Environmental Protection Agency

EPEC .....Enteropathogenic *Escherichia coli*

ETEC .....Enterotoxigenic E*scherichia coli*

FFF .....Field-flow fractionation

FS .....Final settler

H_2O_2.....Hydrogen peroxide

HAB .....Harmful algae bloom

HC .....Hemorrhagic colitis

HGMS .....High gradient magnetic separation

HPLC .....High-performance liquid chromatography

HRP .....Horseradish peroxidase

HUS .....Hemolytic uremic syndrome

ICP.....Ion concentration polarization

ID .....Inflow dam

IL .....Ionic liquid

IR.....Infrared

ISH .....In situ hybridization

ISO .....International Organization for Standarization

IT .....Integration time

LB.....Luria-Bertani

LD.....Legionnaires' disease

LED .....Light-emitting diode

LFICA.....Lateral flow immunochromatographic assay

LIF .....Laser-induced fluorescence

LoD .....Limit of detection

LoQ .....Limit of quantification

L. pneumophila.....*Legionella pneumophila*

µFMS .....Microfluidic magnetic separation

µPAD .....Microfluidic paper-based analytical device

mAb .....Monoclonal antibody

MCE.....Microchip capillary electrophoresis

MF .....Microfiltration

MIL .....Magnetic ionic liquids

MNP.....Magnetic nanoparticle

NC .....Nitrocellulose

OD .....Optical density

OF.....Outflow fish

pAb.....Polyclonal antibody

PBS.....Phosphate buffered saline

PhC.....Phycocyanin

PC.....Polycarbonate

PCB .....Printed circuit board

PCR .....Polymerase chain reaction

PE.....Phycoerythrin

PMMA .....Polymethyl methacrylate

PSA.....Pressure sensitive adhesive

PVDF .....Polyvinylidene difluoride

qPCR.....Quantitative polymerase chain reaction

RFU .....Relative fluorescence value

RI.....Refractive index

RNA .....Ribonucleic acid

RT-PCR.....Reverse transcription polymerase chain reaction

SAP.....Superabsorbent polymer

SELEX .....Systematic evolution of ligands by exponential enrichment

Sg .....Serogroup

SNR .....Signal to noise ratio

SPE.....Screen-printed electrodes

SRP.....Surface plasmon resonance

STEC .....Shiga toxin-producing *Escherichia coli*

TMB.....3,3',5,5'-Tetramethylbenzidine

Tw-20 .....Tween-20

TX-100.....Triton 100

UV .....Ultraviolet

VBNC.....Viable but non-culturable

WBR .....Western blocking reagent

WHO .....World Health Organization

Units:

° .....Angle degree

°C .....Celsius degree

$CFU \cdot mL^{-1}$ .....Colony forming units per milliliter

h .....hour

lx .....Lux

$\mu g \cdot mL^{-1}$ .....Microgram per milliliter

µL .....Microliter

µm .....Micrometer

mA .....Milliampers

mL .....Milliliter

$mL \cdot min^{-1}$ .....Milliliters per minute

$mL \cdot s^{-1}$.....Milliliters per second

mm .....Millimeter

mm^2.....Millimeter square

min .....Minute

M .....Molar concentration

ms .....Millie second

nm .....Nanometer

rpm.....Revolutions per minute

SUMMARY /RESUM /LABURPENA /RESUMEN

SUMMARY

Microbial water quality monitoring aims to protect consumers from diseases caused by pathogens transmitted by ingestion, aspiration (air transmission), or contact with contaminated waters. In the last decades, biosensor technology has been postulated as one of the most promising alternatives to substitute the tedious, bulky, and long-time requiring conventional methods. Nevertheless, most biosensors lack the ability to process large water volumes required for current microbial water quality regulations, which is translated into high detection limits. In this regard, the development of devices able to integrate the concentration of bacteria present in large water volumes, and their detection becomes an issue of relevance.

This thesis presents the development of a device able to use microfiltration membranes as support for both processes, concentration, and detection. The device consists of a flexible concentration platform, which is modified according to the detection needs of the target bacteria. Herein, three different microorganisms have been selected as the work scenarios for the concentration/detection prototype. *Escherichia coli* has been selected for its extensive use as indicator of fecal pollution, *Legionella pneumophila*, for its health importance and *Shynechocystis sp.* as model cyanobacterium for their health and environmental impact.

As is the case of most bacteria, *Escherichia coli* and *Legionella pneumophila* require labeling in order to make them detectable. Thus, an on-filter immunoassay has been developed, employing the membrane used for concentration, also as the support for the immunodetection. The biggest drawback of using microfiltration membranes as support for immunodetection is the nonspecific binding of antibodies to the membrane, consequently giving false positives. Thus, different membrane materials, membrane blocking reagents, and antibody washings have been tested to find the best combination for both *E. coli* and *Legionella* fast detection.

The immunodetection is carried out using antibodies enzymatically labeled with horseradish peroxidase and 3,3',5,5'-Tetramethylbenzidine (TMB) as substrate. Hence, as TMB is widely used either as colorimetric or redox substrate, it has been demonstrated that the developed on-filter immunoassay can be coupled to both absorbance and chronoamperometric measurements. The whole assay

takes 2 h reducing significantly the time needed by conventional methods (2-10 days) and providing a detection limit lower than 10 CFU·mL^{-1}, which could permit achieving the limits established by regulation standards.

The developed device has also been adapted to detection of cyanobacteria. Cyanobacteria contain pigments able to absorb light and emit fluorescence naturally, acting as intrinsic bioreceptors. Moreover, the photosynthetic pigment phycocyanin (PhC) allows to distinguish cyanobacteria from eukaryotic algae. Therefore, the concentration device has been provided with optical elements required for on-filter cyanobacteria detection. A light-emitting diode (LED) to excite PhC and a detector that collects its fluorescence emission have been implemented, in addition to integrating the electronics necessary for their control. The developed system is able to detect cyanobacteria concentrations within the vigilance level established by WHO (<500 cell·mL^{-1}) in less than 10 min even in real samples. Moreover, the use of low cost miniaturized optical components has allowed the design and development of a portable and straightforward prototype suitable for fast and *in-situ* detection and quantification of cyanobacteria in water samples.

The performance and versatility shown by the microbial detection platform designed and tested in this thesis allows us to envision this new technology as a viable alternative towards a fast and cost-effective detection system for microbial water quality monitoring.

RESUM

La monitorització de la qualitat microbiana de l'aigua té com a objectiu protegir els consumidors de malalties causades pels patògens transmesos per ingestió, aspiració (transmissió d'aire) o contacte amb aigües contaminades. En les darreres dècades, la tecnologia dels biosensors s'ha postulat com una de les alternatives més prometedores per substituir els mètodes convencionals tediosos, voluminosos i que requereixen de llargs temps. No obstant això, la majoria de biosensors no tenen la capacitat de processar els grans volums d'aigua necessaris per a la regulació de la qualitat microbiana, que es tradueix en límits alts de detecció. En aquest sentit, el desenvolupament de dispositius capaços d'integrar la concentració de bacteris presents en grans volums d'aigua i la seva detecció esdevé un tema rellevant.

Aquesta tesi presenta el desenvolupament d'un dispositiu capaç d'utilitzar membranes de microfiltració com a suport de tots dos processos, el de concentració i el de detecció. El dispositiu consta d'una plataforma de concentració modelable, que es pot modificar segons les necessitats de detecció del bacteri objectiu. En aquet treball, s'han seleccionat tres microorganismes diferents com a possibles escenaris de treball del prototip de concentració i detecció. *Escherichia coli* ha estat seleccionada per la seva àmplia utilització com a bacteri model de la contaminació fecal, *Legionella pneumophila*, per la seva importància per a la salut i *Shynechocystissp*. com a cianobacteri model per el seu impacte en la salut i al medi ambiental.

Com és el cas de la majoria de bacteris, cal que *Escherichia coli* i *Legionella pneumophila* siguin etiquetats per detectar-los. Així, s'ha desenvolupat un immunoassaig sobre filtre que empra la membrana utilitzada per a la concentració, i també com a suport per a la immunodetecció. Tot i això, l'inconvenient més gran d'utilitzar membranes de microfiltració com a suport per a la immunodetecció és la unió inespecífica dels anticossos a la membrana, donant per tant, falsos positius. Així, s'han provat diferents materials de membrana, reactius de bloqueig de membrana i rentats d'anticossos per trobar la millor combinació per a la detecció ràpida de *E. coli* i *Legionella*.

La immunodetecció es realitza mitjançant anticossos marcats enzimàticament amb horeseradish peroxidasa i 3,3',5,5'-Tetrametilbenzidina (TMB) com a substrat. Per tant, com el TMB es àmpliament utilitzat com a substrat

colorimètric o redox, s'ha demostrat que el immunoassaig sobre filtre es pot combinar tant a mesures d'absorbància com a de cronoamperomtría. L'assaig complet triga 2 h reduint significativament el temps necessari pels mètodes convencionals (2-10 dies) i obtenint límits de detecció per sota dels 10 CFU·mL^{-1}, cosa que podria permetre assolir els límits establerts per les normes de regulació.

El dispositiu desenvolupat també s'ha adaptat a la detecció de cianobacteris. Els cianobacteris contenen pigments capaços d'absorbir llum i emetre fluorescència de manera natural, actuant com a bioreceptors intrínsecs. D'altra banda, el pigment fotosintètic ficocianina (PhC) ofereix l'especificitat per a distingir-los de les algues eucariotes. Per tant, el dispositiu de concentració s'ha dotat d'elements òptics per a la detecció de cianobacteris sobre el filtre. S'ha implementat un díode emissor de llum (LED) per excitar la PhC i un detector que recull la seva emissió de fluorescència, a més d'integrar l'electrònica necessària per al seu control. El sistema desenvolupat és capaç de detectar concentracions de cianobacteris dins del nivell de vigilància (<500 cèl·lules·mL^{-1}) establert per l'OMS en menys de 10 minuts fins i tot en mostres reals. A més, l'ús de components òptics miniaturitzats de baix cost ha permès obtenir un prototip portàtil i senzill adequat per a l'avaluació ràpida i *in-situ* de cianobacteris.

El rendiment i la versatilitat mostrats per la plataforma per a la detecció microbiana dissenyada i provada en aquesta tesi, ens permet preveure aquesta nova tecnologia com una alternativa viable cap a un sistema de detecció ràpid i rentable per a la supervisió de la qualitat de l'aigua microbiana.

LABURPENA

Uraren kalitate mikrobiologikoaren kontrolaren helburua, kontsumitzaileak kutsatutako urak irentsiz, aspiratuz (aire-transmisioa) edo ukituz transmititzen diren patogenoek eragindako gaixotasunetatik babestea da. Azken hamarkadan, biosentsoretan oinarritutako teknologia, ekipamendu handiak eta denbora luzeak behar dituzten ohiko metodo astunen alternatiba gisa agertu da. Hala ere, biosentsore gehienek ez dute kalitate mikrobiologikoa ebaluatzeko behar den ur-bolumen handiak prozesatzeko gaitasunik, eta horrek euren detekzio-mugak altuak izatea eragiten du. Hori dela eta, ur-bolumen handietan aurkitzen diren bakteriak kontzentratzeko eta detektatzeko gaitasunak integratzeko gai diren gailuen garapena gai garrantzitsu bilakatzen da.

Tesi honek, kontzentrazio zein detekzio prozesuetarako euskarri gisa mikro filtrazio mintzak erabiltzeko gai den gailu baten garapena aurkezten du. Gailua, kontzentrazio-plataforma moldakor bat da, intereseko bakteriak detektatzeko beharren arabera aldatu daitekeena. Kasu honetan, hiru bakteria genero aukeratu dira kontzentrazio/detekzio prototipoaren lan-agertoki gisa. *Escherichia coli,* gorotz-kutsaduraren bakteria eredu gisa asko erabiltzen delako, *Legionella pneumophila,* osasun arloan duen garrantziagatik, eta azkenik *Shynechocystis sp.* zianobakterioen eredu gisa, haiek osasunean eta ingurumenean eragiten duten inpaktuagatik.

Bakteria gehienen kasuan bezala, *Escherichia coli* eta *Legionella pneumophila* bakteriek markaketa beharra dute detektatuak izateko. Hala, mintz-gaineko immunoentsegu bat garatu da, kontzentraziorako erabilitako mintz berbera detekziorako euskarri gisa erabiliz. Baina, immunodetekziorako euskarri gisa kontzentraziorako erabilitako mintz berbera erabiltzeak, eragozpen bat du, antigorputzak modu ez-espezifikoan mintzei lotzea, ondorioz, positibo faltsuak eraginez. Hala, hainbat mintz material, mintzak blokeatzeko erreaktibo eta antigorputz garbiketa ezberdin probatu dira *E. coli* eta *Legionella*-ren detekzio azkar baterako konbinaziorik onena aurkitu nahian.

Immunodetekzioa egiteko, entzimatikoki horseradish peroxidasarekin etiketatutako antigorputzak erabili dira eta 3,3', 5,5'-Tetramethylbenzidine (TMB) substratu gisa. Beraz, TMB-a substratu kolorimetriko edo redox gisa asko erabiltzen denez, mintz-gaineko immunoentsegua absorbantzia zein kronoamperometria neurketetara akopla daitekeela frogatu da. Saiakuntza

osoak 2 h behar ditu, metodo konbentzionalen bidez (2-10 egun) behar den denbora nabarmen murriztuz eta 10 $CFU \cdot mL^{-1}$ baino gutxiagoko detekzio-mugak lortuz. Horri esker, ur-bolumenekin jokatuz, erregulazio-arauetan ezarritako mugak lor daitezke.

Garatutako gailua zianobakterioen detekziorako ere egokitu da. Zianobakterioek argia xurgatzeko eta fluoreszentzia emateko gaitasuna duten pigmentuak dituzte, berezko bioerrezpetore moduan jokatuz. Gainera, Fikozianina (PhC) pigmentu fotosintetikoak, eurak alga eukariotoetatik bereizteko aukera eskaintzen du. Hori dela eta, kontzentrazio-gailua elementu optikoz hornitu da mintz gainean harrapatutako zianobakterioak detektatzeko. Diodo argi-igorle bat (LED) ezarri da PhC-na kitzikatzeko, eta detektagailu bat, hark igorritako fluoreszentzia-emisioa jasotzeko, baita hauek kontrolatzeko behar den elektronika ere. Garatutako sistema, 10 minutu baino gutxiagoan Munduko Osasun erakundeak ezarritako zaintza-mailaren barruan (<500 $zelula \cdot mL^{-1}$) dauden zianobakteria-kontzentrazioak detektatzeko gai da, baita lagin errealetan ere. Gainera, kostu txikiko osagai optiko miniaturizatuaren erabilerari esker, zianobakterioen ebaluazio azkarra eta lekukoa egiteko aukera eskaintzen duen prototipo eramangarri eta erabil erraz bat lortu da.

Beraz, tesi honetan diseinatu eta probatu den gailuak bakterio desberdinak detektatzeko erakusten duen moldakortasunak, teknologia berri hau uraren kalitate mikrobiologikoa kontrolatzeko detekzio-sistema bizkorrago sinpleago eta errentagarriago baterako irtenbide gisa aurkezten du.

RESUMEN

La monitorización de la calidad del agua tiene como objetivo proteger a los consumidores de enfermedad causadas por patógenos transmitidos por ingestión, aspiración (transmisión por aire), o contacto con aguas contaminadas. En las últimas décadas, la tecnología de biosensores se ha postulado como una de las alternativas más prometedoras para sustituir los métodos convencionales tediosos, voluminosos y que requieres largos tiempos de ensayo. Sin embargo, la mayoría de los biosensores carecen de la capacidad para procesar los grandes volúmenes de agua requeridos por las regulaciones actuales establecidas para calidad microbiana del agua, lo que se traduce en altos límites de detección. En este sentido, el desarrollo de dispositivos capaces de integrar la concentración de bacterias presentes en grandes volúmenes de agua, y su detección se convierte en un tema de relevancia.

Como es el caso de la mayoría de las bacterias, *Escherichia coli* y *Legionella pneumophila* requieren ser marcados para ser detectables. Por lo tanto, se ha desarrollado un inmunoensayo en el filtro, que emplea la membrana utilizada para la concentración, también como soporte para la inmunodetección. El mayor inconveniente del uso de membranas de microfiltración como soporte para la inmunodetección es la unión inespecífica de anticuerpos a la propia membrana, dando como resultado falsos positivos. Por lo tanto, se han probado diferentes materiales de membrana, reactivos de bloqueo de membrana y lavados de anticuerpos para encontrar la mejor combinación para la detección rápida de *E. coli* y *Legionella.*

La inmunodetección se lleva a cabo utilizando anticuerpos marcados enzimáticamente con horseradish peroxidasa (HRP) y 3,3',5,5'-tetrametilbencidina (TMB) como sustrato. Por lo tanto, como TMB se usa ampliamente como sustrato colorimétrico o redox, se ha demostrado que el inmunoensayo desarrollado en el filtro se puede acoplar tanto a medidas de absorbancia como de cronoamperometría. El ensayo completo lleva 2 h, reduciendo significativamente el tiempo necesario para los métodos convencionales (2-10 días) y proporcionando un límite de detección inferior a 10 UFC·mL^{-1}, lo que podría permitir alcanzar los límites establecidos por los estándares de regulación.

El dispositivo desarrollado también se ha adaptado para la detección de cianobacterias. Las cianobacterias contienen pigmentos capaces de absorber la luz y emitir fluorescencia de forma natural, actuando como biorreceptores intrínsecos. Además, el pigmento fotosintético ficocianina (PhC) permite distinguir las cianobacterias de las algas eucariotas. Por lo tanto, el dispositivo de concentración se ha provisto de elementos ópticos necesarios para la detección de cianobacterias en el filtro. Se ha implementado un diodo emisor de luz (LED) para excitar la PhC y un detector que recolecta su emisión de fluorescencia, además de integrar la electrónica necesaria para su control. El sistema desarrollado puede detectar concentraciones de cianobacterias dentro del nivel de vigilancia establecido por la OMS (<500 células·mL^{-1}) en menos de 10 minutos, incluso en muestras reales. Además, el uso de componentes ópticos miniaturizados de bajo coste ha permitido el diseño y desarrollo de un prototipo simple y portátil para la detección y cuantificación rápida e *in-situ* de cianobacterias en muestras de agua.

El rendimiento y la versatilidad demostradas por la plataforma de detección microbiana diseñada y probada en esta tesis, nos permite pensar en esta nueva tecnología como una alternativa viable hacia un sistema de detección rápido y rentable para la monitorización de la calidad microbiana del agua.

1. INTRODUCTION

1.1 WATER MICROBIAL QUALITY AND HEALTH

Water is essential to sustain life and safe water supply should be universally available to all. Water may contain pathogenic organisms, which include bacteria, viruses, protozoa, and helminths (Table 1), and waterborne diseases related to these pathogens are a worldwide concern [1,2].

Even though since the 20th century waterborne outbreaks have decreased radically, it is estimated that 3.4 million people, mostly children, die each year and higher numbers of illnesses are reported every day due to the microbial contamination of water used for drinking, recreation, irrigation and aquaculture [3,4]. It is calculated that 2 billion people drink water that is contaminated by feces and 4.5 billion use a sanitation system that does not adequately protect either their family or the downstream community from harm [5].

Major waterborne diseases include diarrhea, cholera, shigellosis, typhoid fever, hepatitis A and E, and poliomyelitis. Between them, diarrheal diseases are 3.6% of the global burden of disease and responsible for 1.5 million deaths (2012) [6]. Most of these death cases occur in low-income countries due to poor water sanitation and hygiene, but they also remain a constant threat even in wealthier nations[4]. Although the simple use of chlorine disinfection can eliminate the majority of microorganisms [7], treatment failures or contamination after treatment are common [2,4,8] and water can be distributed and consumed before routine microbial controls reveal the presence of pathogens [9,10]. Thus, timing is essential in pathogen detection and the delay or inaccurate diagnosis of the pathogens are the primary cause of mortality and illness [11].

Microbial water quality measurements aims to protect consumers from illness due to infections caused by waterborne pathogens ingestion, air transmission, or contact [12,13]. There are several methods for testing the microbial quality of water through indicator organisms. The two most common assays are the membrane filtration and the multiple-tube fermentation method. The membrane filtration method consists of filtering water samples and placing the filters on selective culturing mediums and counting colonies, while multiple-tube fermentation involves adding specific quantities of the sample on tubes containing a nutrient broth and looking for the development of gas and/or turbidity that the bacteria produce. The presence or absence of gas in each tube is used to calculate an index known as the Most Probable

Number [14]. However, in spite of our best efforts to monitor water quality, outbreaks continue to occur.

Table 1. Pathogens in drinking water system and their related diseases.**

Group	***Pathogen***	***Disease caused***
Bacteria	*Burkholderia pseudomallei*	Melioidosis
	Campylobacter spp., C. jejuni	Diarrhea
	Cyanobacteria *(Mycrocystis, Anabaena, Aphanizomenon)*	Diarrhea, toxin production
	***Escherichia coli* – pathogenic**	Diarrhea
	E. coli O157:H7	Diarrhea, hemolytic-uremia
	Legionella pneumophila	Legionnaire's disease (pneumonia)
	Non-tuberculous mycobacteria	Pulmonary disease, skin infection
	Pseudomonas aeruginosa	Pulmonary disease, skin infection
	Salmonella typhi	Typhoid dysentery, diarrhea
	Salmonella enterica	Salmonellosis, diarrhea
	Shigella spp.	Shigellosis, diarrhea
	Vibrio cholerae	Diarrhea, Cholera
	Yersinia enterocolitica	Diarrhea
Viruses	Adenoviruses	Diarrhea, respiratory infection
	Enteroviruses	Diarrhea, respiratory infection
	Poliovirus	Poliomyelitis
	Coxsackievirus	Meningitis
	Astroviruses	Diarrhea
	Hepatitis viruses A, E	Hepatitis
	Noroviruses	Diarrhea
	Sapoviruses	Diarrhea
	Rotavirus	Diarrhea
Protozoa	*Acanthamoeba spp.*	Keratitis, encephalitis
	Cryptosporidium spp.	Cryptosporidiosis
	Cyclospora cayetanensis	Diarrhea
	Entamoeba histolytica	Amoebic dysentery
	Giardia lamblia	Giardiasis (Beaver fever)
	Naegleria fowleri	Primary amoebic meningoencephalitis
	Toxoplasma gondii	Toxoplasmosis
Helminths	*Dracunculus medinensis*	Dracunculiasis (Guinea worm disease)
	Schistosoma spp.	Schistosomiasis

** Adapted from table 7.1 in WHO Guidelines for drinking water quality, with data obtained from Ramírez-Castillo et al. [1], Gerba & Pepper [2], Lebaron et al. [20].

1.2 BACTERIA ASSOCIATED WITH WATERBORNE DISEASES

Bacteria are prokaryotic and unicellular microorganisms ubiquitous in aqueous environments [2,15]. Most of the bacterial pathogens are enteric bacteria originated in the intestinal tract of humans and warm-blooded animals entering the water via fecal contamination [16]. However, microbial water safety is not only related to fecal contamination. Some waterborne bacteria, such as *Legionella, Burkholderia pseudomallei* and different mycobacteria, can grow in natural freshwater and colonize water distribution networks [16,17].

Evaluation of waterborne pathogens is based on their virulence or potential for causing disease in humans, which is related to the infective dose [18]. The infective dose is the number of organisms required to cause infection and tends to vary considerably depending on the bacterial pathogen. For example, the median infectious dose for most enteric species as *Yersinia, Salmonella,* and *Escherichia coli (E. coli)* are high, in the range of 10^6-10^8 cells, whereas other bacterial species as *E. coli O157:H7*, *Campylobacter,* and *Shigella,* have low infectious doses of hundred cells (or less) [19,20].

1.2.1 Enteric bacteria

The existence of some enteric bacterial pathogens has been known for more than a hundred years. In the 19th century, *Vibrio cholerae* and *Salmonella enterica* were the first waterborne pathogens to be recognized. Since then, many other clinically significant species have been identified. The main enteric bacteria belong to the genera *Salmonella, Shigella, Campylobacter, Yersinia, Escherichia*, and *Vibrio* [20].

Fecal or enteric bacteria, are bacteria able to multiply in the digestive tract of humans or warm-blooded animals and are typical members of the intestinal microbiota [15]. The transmission occurs mainly by the ingestion of either drinking or recreational fecal contaminated water and generates gastrointestinal illnesses.

Most waterborne bacterial pathogens enter aquatic environments via fecal contamination. When these microorganisms are introduced in water, the environmental conditions are very different, and therefore, their ability to reproduce and survive is limited. Because its detection and counting at the laboratory level are slow and laborious, a group of indicators is used for faster and easier detection. The

most used group is fecal coliform bacteria, and the estimate of *Escherichia coli* concentration is the most common approach to assess the extent of microbial pollution in water. According to the WHO the *E. coli* concentration from drinking water as well as recreational waters should be zero Colony Forming Units (CFU) per 100 mL [21,22]. Nevertheless, the correlation between the absence of indicators and waterborne pathogens can be questionable [12,23] since, although to a lesser extent, diseases still occur in the absence of indicators [24,25].

Escherichia coli

Escherichia coli (*E. coli*) is a rod-shaped, gram-negative bacterium that measures approximately 0.5 µm in width by 2 µm in length. It is a predominant facultative anaerobe of the human colonic microbiota and typically colonizes the gastrointestinal tract of human infants within hours after birth. Initially, it was believed that *E. coli* was unable to survive for long periods in water environments. Nevertheless, studies have shown its ability to both survive and replicate in the environment and the waterborne transmission of pathogenic *E. coli* strains has been well documented for contaminated recreational and drinking waters [26–28].

Several strains of *E. coli* have acquired specific virulence attributes being able to cause a broad spectrum of diseases. The pathotypes referred to as Shiga toxin-producing *E. coli* (STEC), including enterohaemorrhagic *E. coli* (EHEC), can cause bloody diarrhea as well as potentially fatal human diseases, such as hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC). *E. coli* O157:H7 is the most recognized serotype of EHEC, and causes many outbreaks of food and waterborne illness. Enteropathogenic *E. coli* (EPEC) is one of the major causes of watery diarrhea in infants, especially in developing countries. Enterotoxigenic *E. coli* (ETEC) is the leading cause of traveler's diarrhea and, enteroaggregative *E. coli* (EAEC) can cause persistent diarrhea, lasting for more than two weeks. Enteroinvasive *E. coli* (EIEC) is genetically, biochemically and pathogenically closely related to *Shigella* [2,26–28].

The infective dose of *E. coli* varies between these strains. Enteropathogenic, enterotoxigenic and enteroaggregative *E. coli* strains require large numbers to cause diarrhea (> 10^6 cells). In contrast, enteroinvasive and enterohemorrhagic strains are able to cause disease with infective dose lower than 100 cells or even lower than 10 cells for the specific case of O157:H7 [29].

1.2.2 Non-fecal bacteria

Some other bacteria, common inhabitants of water environments, also have the potential to be transmitted through water and cause diseases [20,30]. They are transmitted by inhalation of water aerosols or by contact during bathing and cause infections occurring in the respiratory tract, skin or even the brain [16,17].

A wide variety of species from the genera *Legionella*, *Pseudomonas, Aeromonas, Klebsiella, Flavobacterium, Enterobacter, Citrobacter, Serratia, Acinetobacter, Proteus, Providencia* and *Mycobacterium,* and *Nocardia* belong to this group [20]. Among them, *Legionella* is one of the non-fecal pathogens with the highest public health impact [30].

Legionella

Legionella is a gram-negative rod-shaped bacterium. It has a typical length between 2 and 5 µm, but when cultured, it appears as long filaments of 10-25 µm[15]. *Legionella* is ubiquitous in natural aquatic habitats, such as lakes, ponds and rivers, where it occurs at relatively low concentrations [16,28]. However, *Legionella* is able to colonize human-made water systems where it can grow unchecked and be transmitted to humans by inhalation or micro-aspiration of aerosols generated by showers, faucets, cooling towers or whirlpool spas [31–33]. *Legionella* is considered a respiratory pathogen and its inhalation leads to two forms of disease: Legionellosis also named Legionnaires' disease (LD), which is a severe respiratory illness involving pneumonia, and Pontiac fever, which is a milder flu-like illness without pneumonia [1,15,23].

Within the genus *Legionella*, at least 50 different species have been identified comprising 70 distinct serogroups. Although all *Legionella spp.* are considered potentially pathogenic for humans, *L. pneumophila* is responsible for 80% to 90% of the identified cases of Legionellosis, particularly serogroup 1 accounts for 50-75% of the cases [16,28,34,35]. Even though the infective dose has not been determined reliably and varies with host susceptibility, pathogenic species of *Legionella,* are especially troublesome for immunocompromised individuals, smokers, young children and elder people (over 60 years) [15,20,35]. For this reason, its occurrence in hospital water-distribution systems and medical equipment such as respirators,

inhalers, and humidifiers is primarily troublesome with mortality rates of 15% to 30% [34].

Spain, France, Germany and Italy are the countries with the highest number of reported cases of LD in Europe [36]. Up to date, there is no common European nor worldwide regulation on the presence of *Legionella* in water. Nevertheless, many countries have developed their own guidelines or regulations [37]. In Spain, the presence of *Legionella* in the water system is legislated by the BOE (RD 865/2003) that establishes that corrective actions must be taken whenever *Legionella* levels exceed 100 $CFU \cdot L^{-1}$ [38].

1.2.3 Harmful Algae Blooms (HABs)

Besides the common waterborne pathogens, other organisms can constitute a risk for human health. An example are microscopic algae (phytoplankton) as a consequence of the ability of some of their members to produce toxins. Thus, events of high concentration of suspended phytoplankton, usually referred to as "algal blooms", may constitute a serious threat that limits the water use for recreational activities as well as for drinking [4]. Many species of phytoplankton, such as dinoflagellates and diatoms, can proliferate intensively in eutrophic waters. However, more incidents have been related to blue-green algae, cyanobacteria, which are able to form extremely high dense surface scums more frequently than eukaryotic algae [39].

Cyanobacteria

Cyanobacteria are gram-negative photoautotrophic prokaryotes able to perform oxygenic photosynthesis. In terms of cellular structure, they have a typical prokaryotic cell organization with a peptidoglycan wall and a cell membrane and without nucleus or organelles (Figure 1.1). However, as in eukaryotic algae and higher plants, they possess a two-photosystem process (Photosystem I and Photosystem II) responsible for oxygenic photosynthesis [40–42]. In addition to chlorophyll-a (Chl-a) and carotenoids also present in eukaryotic algae, cyanobacteria have accessory pigments such as phycocyanin (PhC) and phycoerythrin (PE) embodied in phycobilisomes present on the thylakoids located in the cytoplasm. These are able to absorb light effectively between the absorption peaks of chlorophyll-

a and carotenoids [43]. Nevertheless, while PhC and Chl-a are found in all cyanobacteria, PE is specific to red cyanobacteria, which are mainly found in marine environments [42].

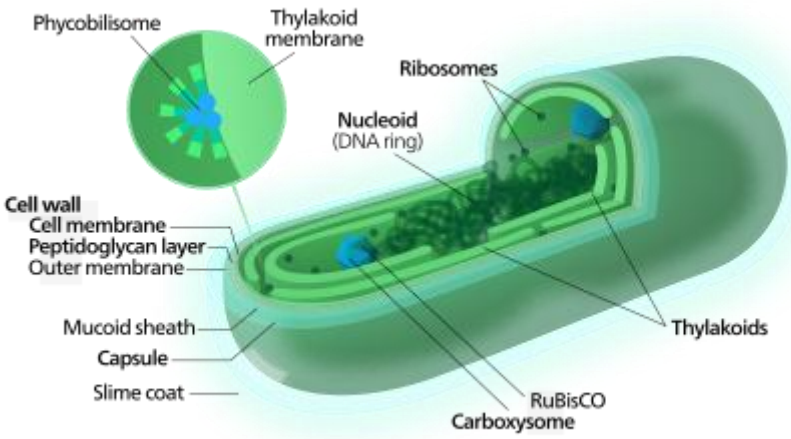

Figure 1.1. Diagram of a cyanobacterium. By Kelvinsong - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=24126121

Generally, cyanobacteria cell sizes range from 1-2 µm (unicellular) to 30 µm (multicellular). Their basic morphology comprises unicellular, colonial, and multicellular filamentous forms. Their ability to photosynthesize using water as the electron donor allows cyanobacteria to colonize a wide range of environments being natural members of lakes, streams, ponds, and other water surfaces [41,43]. Moreover, when light intensity, nutrient availability (especially phosphorus), water temperature, pH, water flow, and water column stability conditions are favorable, cyanobacteria can rapidly multiply in surface water and cause blooms [41,44].

Accumulation of cyanobacteria in blooms may lead to excessive oxygen consumption, which causes aquatic animal death by hypoxia [40,45], as well as to the generation of adverse tastes and odors in water [46,47]. Notwithstanding, the most problematic issue about cyanobacteria is their ability to produce a wide range of allergenic, toxic, or carcinogenic substances as secondary metabolites that are hazardous for animal and human health [48–53]. These cyanotoxins include neurotoxins, hepatotoxins, and dermatoxins [44].

In most cases, these toxins are intracellular and are released when the cells die or their membrane breaches. Thus, it has been reported that conventional water

treatment methods increase toxin concentration in drinking water due to cell lysis produced by the collection and treatment activities. Exposure to cyanobacteria and their toxins can happen by inhalation, direct contact during recreational activities or by intake of drinking water contaminated with cyanotoxins released during treatment [44,50].

For cyanobacteria related health risk control, the World Health Organization (WHO) established three alert levels based on cell concentration and the related health risk [43]: (1) Vigilance level, corresponds to between 500 and 2000 cells·mL^{-1} and constitutes the earliest possible stage of bloom development. (2) Alert Level 1 (≥2000 and <100000 cells·mL^{-1}), when the cyanobacteria biomass is enough to produce high concentrations of cyanotoxin endangering the suitability of the water for human consumption. (3) Alert Level 2, when the concentration of cyanobacteria is higher than 100000 cells·mL$^{-1,}$ and a toxic bloom with high biomass is established, implying risk for human health.

1.3 (BIO)SENSORS FOR WATER MICROBIAL QUALITY MONITORING

Conventional culture-based methods for bacterial pathogen detection rely on the growth of the target organism on selective culture media. Because they are sensitive, inexpensive, and straightforward, culture-based methods are generally considered the golden standard. However, these methods are also tedious and require several days, depending on the growth ability of bacteria [11]. For example, 2 to 8 days are needed for the detection of pathogenic strains of *E. coli,* and 2 to 10 for *Legionella* [44]. In an attempt to reduce analysis time from days to hours, much more sophisticated methods have been developed based on immunoassays and nucleic acid detection, such as enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and in situ hybridization (ISH) techniques[54–56]. Nevertheless, these methods have several limitations, such as high costs or the need for qualified personnel in addition to bulky benchtop equipment impossible to operate outside the laboratory [55–57].

Therefore, there is a need for alternative bacterial detection systems to develop more rapid and cost-effective point of care devices. In the last decades, (bio)sensors have

gained attention due to the advantages they offer regarding miniaturization, integration, and automation, which can lead to the appearance of advanced, fast and low-cost alternatives for the detection of microbial pathogens.

1.3.1 Definition of (bio)sensors

Biosensors are classified as a sub-set of chemical sensors. Chemical sensors are devices that transform chemical information, ranging from a specific sample component to total composition analysis, into an analytically useful signal [58]. Chemical sensors are constituted by two essential components: a recognition system (receptor) and a physicochemical transducer. When the receptor is a biological sensing element (bioreceptor), these sensors are called biosensors [59,60]. Thus, biosensors are devices that use bioreceptors that interact with the analyte of interest and translate the information from the biochemical domain, into a chemical or physical output signal with a defined sensitivity. The part of the sensor responsible for this signal transfer is called transducer (Figure 1.2) [58,61].

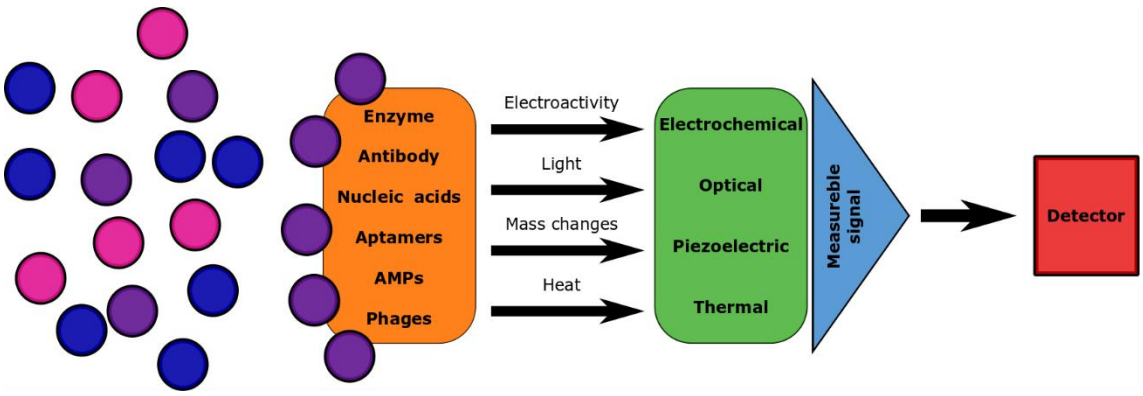

Figure 1.2. General scheme of a biosensor.

In general, biosensors are classified according to the biorecognition element or the transducer used. Bioreceptors can include enzymes, antibodies, aptamers, antimicrobial peptides, phages or even microbial cells. On the other hand, the transductor employed relies on the type of physicochemical changes resulting from the sensing event and can be mass-based, electrochemical, or optical.

1.3.2 Biorecognition elements

The primary purpose of the biorecognition elements is to provide specificity to the biosensor, and this will depend on the affinity between the bioreceptor and the target. There is a wide range of receptors that can be used either of natural (antibodies and enzymes) or of synthetic origin (aptamers) [62].

1.3.2.1 Antibodies

Antibodies (immunoglobulins or Igs) are molecules produced by biological systems in response to the presence of a foreign agent (antigen). They are typically ~150 kDa in size and share the general structure of a "Y" shaped 3D conformation, made up of two heavy (H) chains paired with two light (L) chains linked together by disulfide bonds (Figure 1.3). Each light chain is composed of a constant domain (C) and a variable domain (V). Each heavy chain has three constant domains (CH1, CH2, and CH3) and one variable domain (VH). The variable regions of the heavy and the light chain are the regions of antigen interaction. Thus, each antibody has two identical antigen-binding sites. These two regions are highly variable in sequence and they vary significantly among antibodies. Its 3D structure creates a unique recognition pattern with high specificity and accuracy for the site recognized at the antigen, also known as epitope [62].

There are two categories of antibodies used in biosensors development according to their production mode: monoclonal and polyclonal. Monoclonal antibodies (mAb) are produced in vitro from hybridoma cell lines and consist of identical antibodies that bind to a single epitope. On the other hand, polyclonal antibodies (pAb) are produced in vivo and consist of antibodies with different affinities that bind to a different number of epitopes on the antigen of interest [63].

Antibodies can be manufactured to recognize specific protein and carbohydrate structures, a principle that can be exploited to identify microorganisms. The external membrane of bacteria consists of multiple proteins and carbohydrate molecules, some of them species-specific or strain-specific. Hence, highly selective and sensitive antibodies are available for many pathogens and used to detect the whole pathogen (their surface proteins) or some of their components (lysate, enzymes, toxin, spore, pili). For these reasons, immunological recognition by antibodies continues to be the

most widely used tool for the selective capture and labeling of microorganisms [63,64].

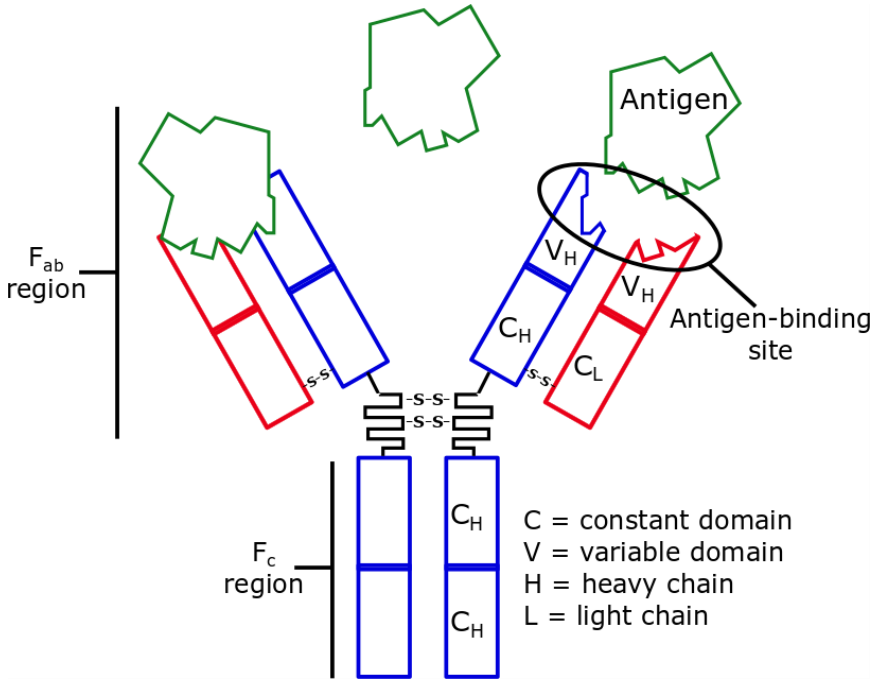

Figure 1.3. Structure of an antibody composed of two identical light (L) chains and two identical heavy (H) chains, which are held together by disulfide bonds to form a flexible Y shape. Each chain is composed of a variable (V) region and a constant (C) region.

1.3.2.2 Enzymes

Almost all enzymes are proteins that acquire the specificity towards the analyte with cavities buried within their 3D structure. The enzymes are chosen based on their specific binding and catalytic activity. When they capture the analyte, this is converted to a measurable product (Figure 1.4). Enzymes, not only offer a high degree of specificity to biosensors, they can also be used for signal amplification. Therefore, in the field of pathogen detection, enzymes are mostly used as labels for other bioreceptors such as antibodies or aptamers [62,65].

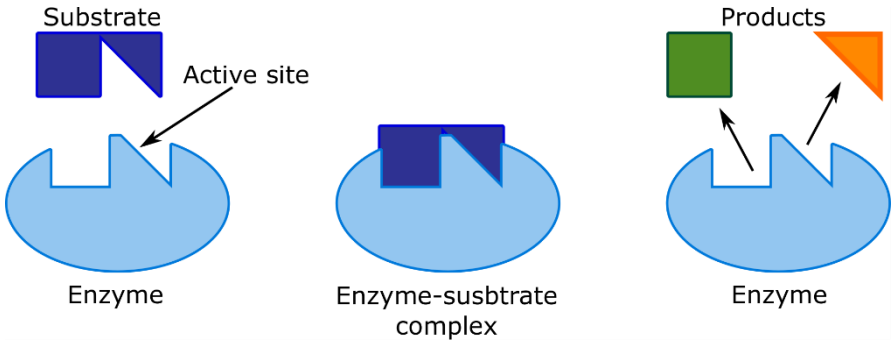

Figure 1.4. Representation of substrate binding to the active site of an enzyme molecule and conversion to product.

1.3.2.3 Nucleic acids

Nucleic acid biosensors, also known as genosensors, detect DNA or RNA from target cells. A DNA fragment complementary to the DNA target sequence is artificially designed and used as a biorecognition element. Specificity is given by the unique complementary recognition pattern between the DNA or RNA bioreceptor and the target sequence [62]. As the nucleic acid content of a cell is low, these types of sensors generally require amplification by PCR or reverse transcription PCR (RT-PCR). Therefore, genosenors for pathogen detection include several steps encompassing lysis, extraction of nucleic acids, purification, and detection, being their integration in a single device challenging [64].

1.3.2.4 Aptamers

Aptamers are small (2-25 kDa) artificial single-stranded DNA or RNA sequences that interact with microbial targets due to the formation of a unique 3D structure. They are produced through consecutive iterative steps of selection and amplification known as the systematic evolution of ligands by exponential enrichment (SELEX). By this production technique, aptamers can be synthesized easily and in large quantities against any target, including targets that are toxigenic or do not produce an immunogenic response. Aptamers present several characteristics, such as thermal and chemical stability, low cost, and low batch-to-batch variation, features that make them a promising alternative to immunological methods [63,66].

1.3.2.5 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are small oligopeptide members of the innate immune system that can kill a wide range of pathogens. They are small oligopeptides that can range from 5 to 100 amino acids and are generally cationic and amphipathic. Their structure confers AMPs the ability to bind to lipidic components (hydrophobic region), to phospholipid groups (hydrophilic region) or to interact with bacterial cell membranes through electrostatic interactions. Hence, AMPs are gaining attention for their use as bioreceptors [67,68].

1.3.2.6 Phages (bacteriophages)

Bacteriophages are viruses that have bacteria as their natural host. They are ubiquitous in almost all environments, where bacteria grow and can survive and replicate even under harsh conditions. Phage amplification only occurs when a viable cell is infected, so phages besides being highly specific offer the advantage of differentiating viable cells. Moreover, phages can be engineered to carry genes for reporter enzymes like alkaline phosphatase that will be expressed during viral replication and before the lysis of the host bacteria. Thus, when the infection cycle is finished, and the phages cause bacterial cell lysis, these enzymes will be released to the media and can be used to facilitate detection (Figure 1.5) [69,70].

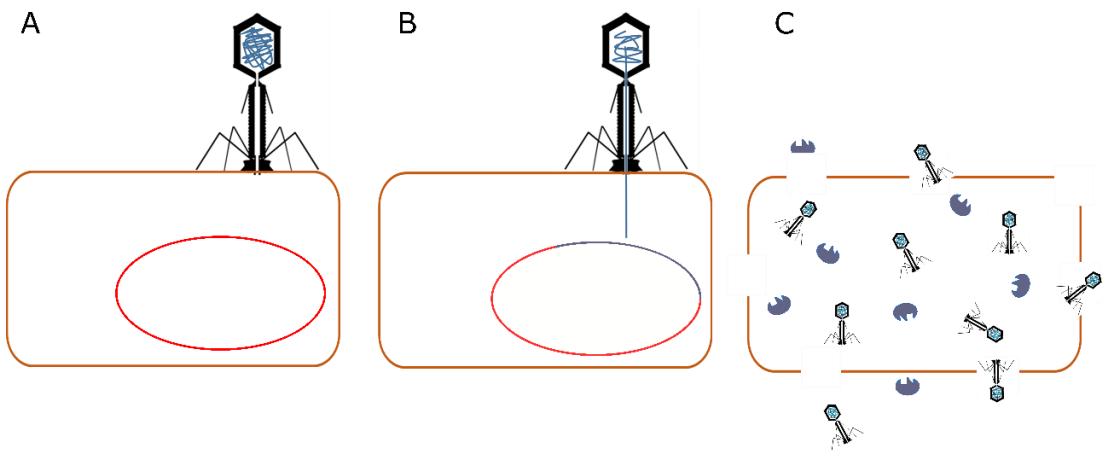

Figure 1.5. Bacteriophage infection cycle. A) Bacteriophage binds to the bacteria through tail spike proteins. B) Bacteriophage infects the cell and inserts its genes and the engineered genes. C) Phages and reporter enzymes are expressed and released.

1.3.3. Transduction methods

The transducer is the part of the sensor responsible for translating the (bio)chemical interaction into a chemical or physical output signal that can be amplified, stored, manipulated, displayed and analyzed [71,72]. Transduction methods are based on electrochemical, optical, piezoelectric and thermal principles. Nevertheless, optical and electrochemical transducers are the predominant technologies for bacterial detection applications and, therefore, they will be discussed more extensively in this thesis (Figure 1.6).

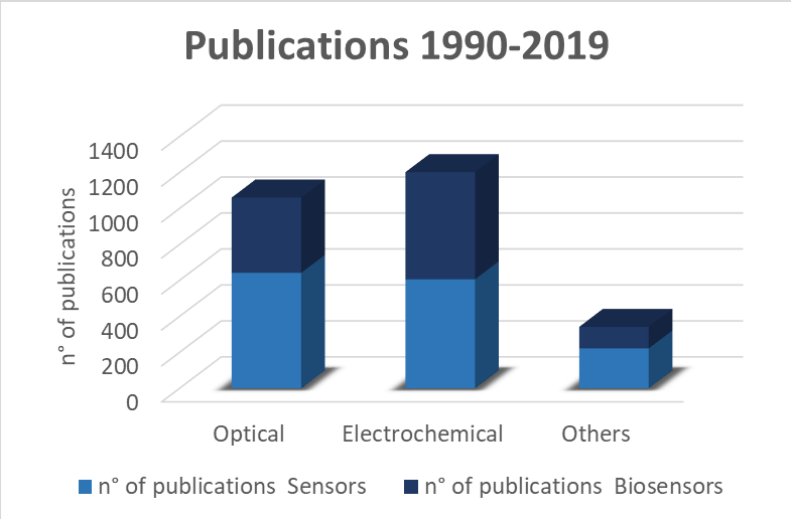

Figure 1.6. Number of publications from 1990 to 2009 on sensors and biosensors based on optical, electrochemical and other (piezoelectric and thermal) transduction methods. Data obtained from Web of Science.

1.3.3.1 Optical biosensors

Optical sensors measure optical changes resulting from the interaction of the analyte with the transducer. These changes in optical properties can affect the absorption, fluorescence, chemiluminescence, light scattering, polarization, Raman scattering, refractive index (Surface Plasmon resonance) or reflection of the sample [59,73,74].

Except for the case of surface plasmon resonance (SPR) based sensors that can detect changes in the refractive index (RI) at the sensor surface [60], most other optical sensors (e.g. absorbance, fluorescence) require the use of optical fibers in various configurations [74]. Due to the advances in the development of low-cost optical fibers during the last decades, they have become an essential part of sensor technology.

Fiber optics based sensors are based on either direct (spectroscopic) or indirect (recognition-based) detection schemes. In direct detection, the intrinsic optical properties of an analyte (color, fluorescence, IR absorption or Raman emission) are measured. On the other hand, in indirect systems, the measured color or fluorescence comes from optically detectable labels or indicator probes [75]. The main points in favor of the use of optical fibers are their excellent light delivery, low cost, and ability not only to excite the target molecules but also to capture the light emitted from the targets [76].

Below, absorbance and fluorescence are explained more in detail as they have been used as transduction methods for bacterial sensing devices developed in this thesis.

Absorbance

Absorbance relies on the absorption of light by biomolecules (Figure 1.7), that is, attenuation of light when it is propagated through a medium. To carry out the measurements, the solution of interest is placed between the light source and the detector. Then, the sample is illuminated and the absorbance spectrum is obtained by a spectrometer measuring light attenuated at various wavelengths. Based on Lambert-Beer law, the amount of light absorbed by the sample is proportional to the concentration of analyte (molecule):

$$A_\lambda = \varepsilon_\lambda \times c \times l \qquad \text{(Equation 1.1)}$$

where absorbance at a particular wavelength (A_λ) is a function of the concentration of the biomolecule (c, units in M), the length of the optical path of the cell or cuvette containing the solution (l, units in cm), and the absorption coefficient (ε_λ, units in $M^{-1} \cdot cm^{-1}$).

Characterization of the transmitted spectrum allows the determination of the concentration of a substance, to carry out kinetic measurements of certain biochemical reactions and to identify some biological species [77,78].

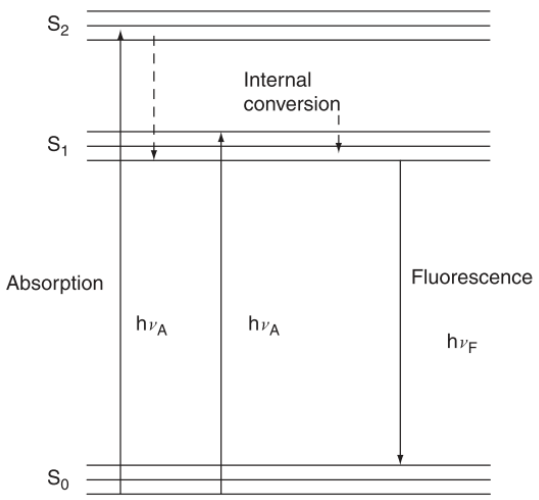

Figure 1.7. Simplified Jablonski diagram depicting the excitation of an electron by absorption of a photon ($h\nu_A$) to higher electronic states S_1 or S_2. The electron returns to the ground electronic state, S_0, from the lowest vibrational state of S_1. Fluorescence is observed with the release of a photon ($h\nu_F$). The dotted and solid arrows in the diagram represent nonradiative and radiative electronic transitions, respectively [78].

Fluorescence

In fluorescence, the absorption of light of a particular wavelength ($h\nu_A$) results in the emission of light in a longer wavelength ($h\nu_F$) (Figure 1.7). When light is absorbed, electrons of the analyte of interest are excited from its ground state (S_0) to an excited state (S_n, n=1,2,...). But the lifetime in the excited state is very short, and the electrons relax back to the ground state (S_0), releasing the excitation energy as fluorescence [79,80]. The emission maximum of the fluorophore occurs at a longer wavelength than the absorption maximum as part of the vibrational energy of the excited state is lost before emission. This energy gap between the absorption maximum and the emission maximum is called the Stoke's shift, and for sensing applications should be large enough to avoid cross-talk between excitation and emission signals (Figure 1.8)[56,78].

As well as absorbance, fluorescence intensity is defined as the product of the molar extinction coefficient, optical path length and solute concentration (Lambert-Beer Law) in addition to the fluorescence quantum yield, the excitation source intensity and fluorescence collection efficiency. Moreover, background noise originated from endogenous samples constituents may be minimized to improve the sensitivity of the detection [81].

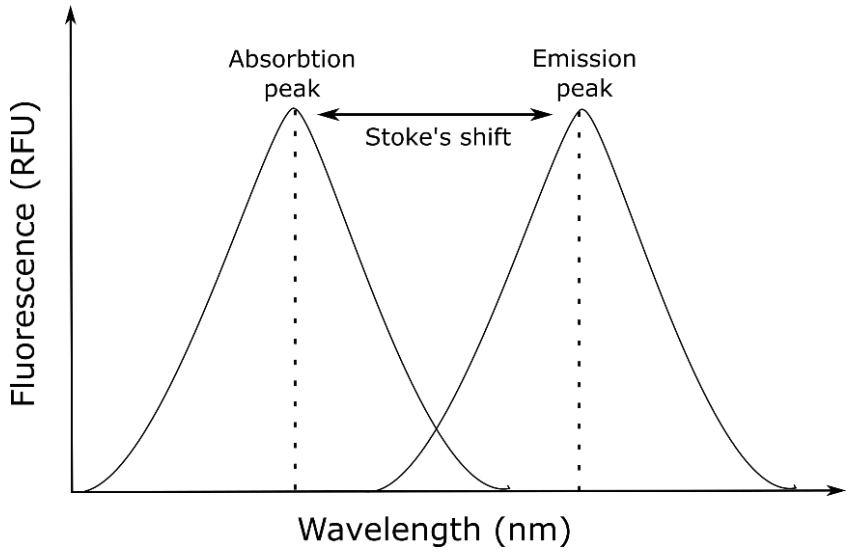

Figure 1.8. Stoke's shift graphical representation.

1.3.3.2 Electrochemical biosensors

Electrochemical biosensors are considered perhaps the most promising technology for the rapid monitoring of microorganisms due to advantages such as simple structure, high sensitivity, low cost, and prompt response. They usually consist of a working electrode, a counter electrode, and a reference electrode. The reference electrode, typically made of silver and silver chloride (Ag/AgCl), is kept at a distance from the reaction site and maintains a known and stable potential. The working electrode acts as the transduction element in the biochemical reaction, and the counter establishes a connection to the electrolytic solution so that the current can be applied in the working electrode. The reaction on the electrode surface is collected and converted to an electrochemical signal proportional to the concentration of the analyte present in the sample. Depending on the electrochemical parameter measured, i.e. potential, impedance or current, electrochemical biosensors can be classified into potentiometric, impedimetric, and amperometric [60,82,83]. In this thesis amperometric sensors are explained more on detail as is one of the transduction method selected for the development of the sensing devices.

Amperometric sensors:

Amperometry is perhaps the transduction method more frequently used for electrochemical biosensors [56,84]. Amperometric biosensors detect current changes resulting from the electrochemical oxidation or reduction of electroactive species at the electrode surface. Measurements are carried out by stepping the potential between the working and the reference electrodes to a point where the generated current changes are directly proportional to the concentration of the analyte [58,83,85].

When amperometric measurements are performed with a stationary electrode in an unstirred solution, this technique is known as chronoamperometry. Under these conditions, mass transport at the surface of the electrode is governed solely by diffusion. When a potential is established at which the target analyte is electrochemically oxidized or reduced, the local analyte concentration drops to zero at the electrode surface ($C_O = 0$). From this moment, the analyte will start to diffuse from the bulk solution of higher concentration to the electrode surface.

The characteristic curve shape of the resulting measurements is the chronoamperogram (Figure 1.9). The current intensity (i) vs time (t) curve of the chronoamperogram reflects the current decay due to a decrease of the analyte concentration in the vicinity of the electrode [83,86]. The values of current intensity as a function of time are defined by the *Cottrell equation*:

$$i = \frac{nFAC^{*}D^{1/2}}{\pi^{1/2}t^{1/2}} \qquad \text{(Equation 1.2)}$$

Where n is the moles of electrons involved in the reaction, F is the Faraday constant, A is the area of the electrode (cm^2), $C^{*}$the concentration of the analyte in the bulk solution ($mol \cdot dm^{-3}$), D is the diffusion coefficient ($cm^{2} \cdot s^{-1}$) and t is time (s). This equation allows relating the registered current with the concentration of the electroactive substance and with its diffusion coefficient.

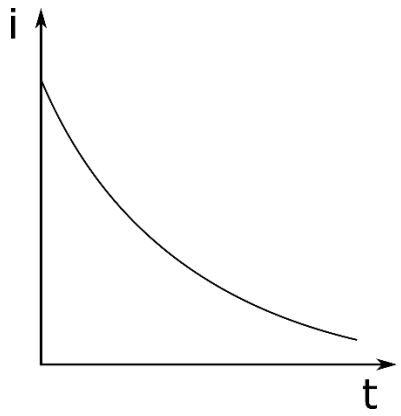

Figure 1.9. Chronoamperometric output signal waveform obeying the Cottrell equation.

1.4 CONCENTRATION SYSTEMS: A SOLUTION TO THE PRE-ENRICHMENT REQUIRED IN WATER MICROBIAL SENSING

The use of biosensors for waterborne pathogen detection has gained attention during the last two decades due to the advantages they offer in terms of miniaturization, integration, fast operation and automation. Nevertheless, the sensitivity and detection limit of these systems has natural limitations [44].

Waterborne pathogens travel through the environment and get diluted to low but clinically concerning concentrations. Thus, in environmental samples, waterborne pathogens are usually present in meager quantities in large water volumes, making their detection very challenging [50,87,88]. For this reason, a sample pre-treatment compatible with the selected detection method should be included in the development of sensors for waterborne bacteria [88].

To allow fast and efficient concentration and separation of bacteria from water, different separation techniques based on magnetic separation, electric separation, centrifugation, size-exclusion filtration, or combinations of them can be used. This section will be focused on the different concentration methods used for pre-concentration or capture of waterborne bacteria for (bio)sensing applications. Thus, a review has been carried out on various works published in the last years describing the development of systems for the concentration and detection of bacteria.

1.4.1 Magnetic capture

In magnetic separation approaches, the target analyte is magnetically isolated from a complex mixture by the application of an external magnetic field. However, most biological compounds and so bacteria, lack intrinsic magnetic properties. Thus, it is necessary to artificially supply magnetic properties to the target microorganism by a magneto-active substrate.

1.4.1.1 Functionalized magnetic surfaces

A particular case of magnetic separation is the use of magnetic nanoparticles. Magnetic nanoparticles are particles available in a wide variety of materials (e.g., iron and nickel) and sizes (50-200 nm). These magnetic nanoparticles can be coated with different bioreceptors with affinity to the target bacteria offering large surface-to-volume ratios for their capture. Thus, the water sample is mixed with the labeled magnetic particles that interact with the bacteria, to trap them for later to apply a magnetic field and concentrate them [44,89,90].

Xue et al. [89] presented a fluorescence-based method that used high gradient magnetic separation (HGMS) to concentrate *E. coli* cells captured by antibody labeled magnetic nanoparticles (MNP). The biosensor was able to handle 10 mL sample volumes with a flow rate of 0.36 $mL \cdot min^{-1}$ (Figure 1.10). Then, immune quantum dots were used to react with MNP-bacteria complexes and detect them by fluorescence. The limit of detection achieved by the biosensor was 14 $CFU \cdot mL^{-1}$ s within 2 h, which made this system promising for the detection *E. coli* presence in water bodies.

More recently, Castillo-Torres et al.[91] presented a proof of concept combining microfluidics with magnetic capture of *E. coli* cells to obtain a microfluidic magnetic separation (μFMS) platform able to process the standard sample size of 100 mL at flow rates up to 120 $\mu L \cdot s^{-1}$. Magnetic microdisks (1.5 μm in diameter) labeled with DNA aptamers were used to obtain microdisk/bacteria complexes that subsequently were passed from the μFMS system. A preliminary study was done using *E. coli* at a concentration of 100 $CFU \cdot mL^{-1}$. Once the microdisk/bacteria complexes were magnetically retained inside the microfluidic platform, fluorescence microscopy was used to confirm their isolation from the water sample. Although the platform showed to be promising, sample incubation with microdisks still needs to be integrated. Additionally, the microscopic evaluation makes the system non-portable.

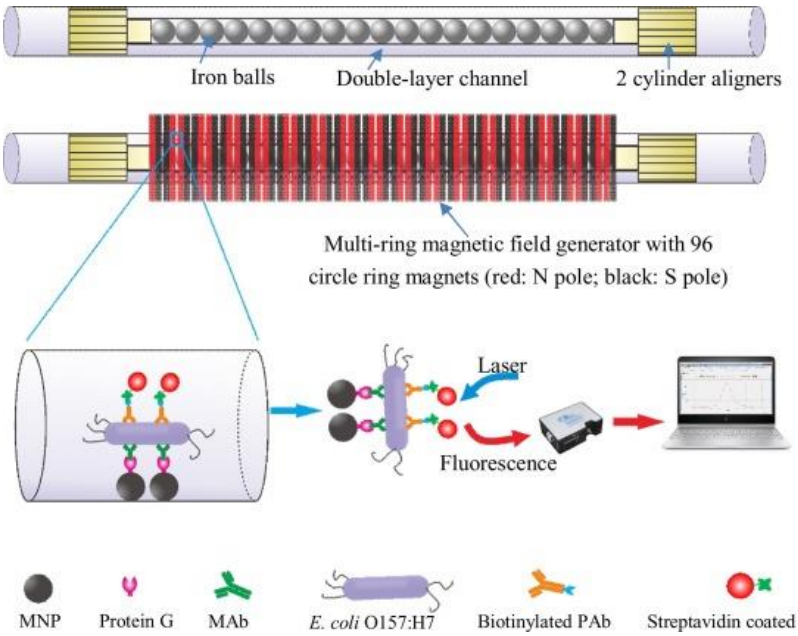

Figure 10. Schematic of the ultrasensitive fluorescence biosensor using a double-layer channel with magnetic nanoparticle and quantum dots for rapid detection of foodborne pathogenic bacteria developed by Xue et al, [89].

Although some assays involving large sample volumes of water and the use of magnetic particles for bacterial capture have been developed as reported above, their application to large sample volumes is not easy. Therefore, many times functionalized magnetic surfaces are used for specific bacterial capture in low sample volumes [70,92] or, as will be discussed afterward, in combination with other concentration techniques for a prior sample volume reduction.

1.4.1.2 Magnetic ionic liquids (MILs)

Ionic liquids (ILs) are a class of molten salts with melting points at or below 100 °C. They are commonly composed of organic cations and organic/inorganic anions, and their structure makes possible for their cationic groups to interact with negatively charged bacterial cells through electrostatic and hydrophobic interaction [93]. When one or more paramagnetic components are incorporated into their structure, a magnetic ionic liquid (MIL) is generated. Thus, MILs possess similar physicochemical properties to conventional ILs (negligible vapor pressures at ambient temperatures and tunable physicochemical properties) in addition to a strong response to external magnetic fields [94–96].

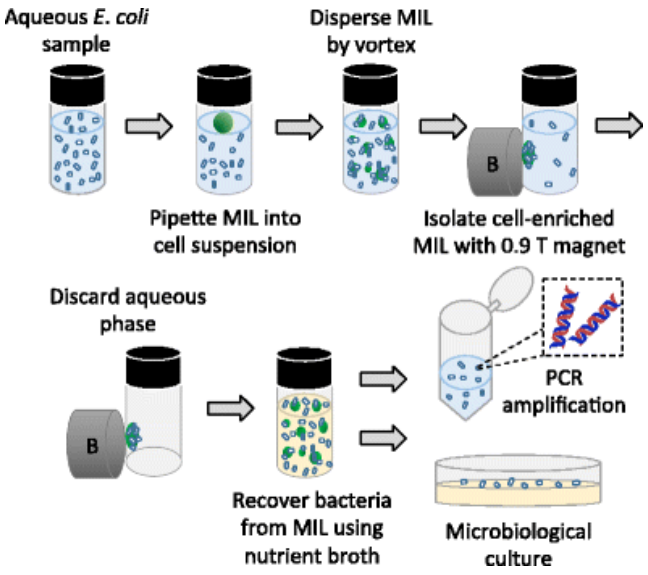

Figure 1.11. Schematic depiction of MIL-based cell extraction developed by Clark et al., [97].

Given that the ILs had already been used for the extraction of bacteria in aqueous matrices, Clark et al., [97] proposed using MILs to capture and isolate *E. coli* bacterial cells (Figure 1.11). They were able to pre-concentrate *E. coli* cells by dispersing a hydrophobic MIL in a 2 mL aqueous sample and detecting concentrations as low as 10^2 CFU·mL^{-1}. The time required for the extraction of bacterial cells was as short as 10 min. Nevertheless, to carry out the subsequent detection, viable cells were cultured or analyzed by qPCR. These methods require large assay times or specific and expensive equipment and therefore, they are unsuitable for integration to biosensors.

Table 1.2. Resume of some experimental parameters of systems using magnetic separation.

Pre-treatment		**Bacteria**	**Vol. (mL)**	**Bioreceptor**	**Transductor**	**LoD (CFU·mL^{-1})**	**Assay t (min)**		**Integration**	**Portability**	**Reference**
							Con.	Detec.			
Magnetic separation	**MPs**	*E. coli*	0.9	Phage	qPCR	100	120		×	×	[70]
		E. coli	0.9	Phage	Absorbance	4.9x10^4	< 120		×	×	[92]
		E. coli	10	Antibody	Fluorescence	14	120		×	✓	[89]
		E. coli	100	Aptamer	Fluorescence microscopy	100	14	-	×	×	[91]
	MILs	*E. coli*	2	-	qPCR	100	10	-	×	×	[97]

1.4.2 Electric separation

This concentration method consists on the capture of charged or polarizable cells by the application of an external electric field. Gram-negative bacteria have lipopolysaccharides covering their cell walls conferring them a strong negative charge. Thus, bacteria can be polarized and captured by methods like ion concentration polarization (ICP), microchip capillary electrophoresis (MCE) or dielectrophoresis (DEP) [44].

1.4.2.1 Ion concentration polarization (ICP)

Ion concentration polarization (ICP) is an ionic transport phenomenon that occurs near an ion-selective membrane (i.e., Nafion) under the application of an external electric field. Due to the flux difference between anions and cations through the membrane, one side becomes depleted of all charged species while the other side is enriched. Hence at the edge of the depleted zone, ions concentrated, thereby creating a concentration band and allowing ICP work as a pre-concentration system [98,99].

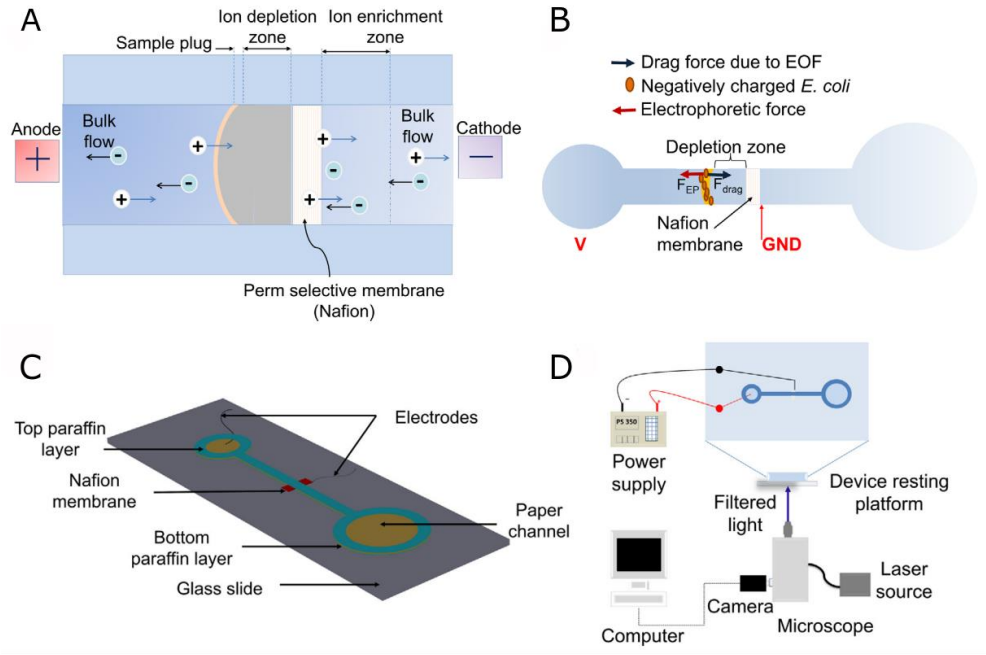

Figure 12. (A) Schematic illustration of ion concentration polarization (ICP) phenomenon at a perm selective membrane; (B) An Illustration of the working principle of ICP for pre-concentration of negatively charge *E. coli* cells on a microfluidic paper-based device; (C) Schematic of the microfluidic paper-based device; (D) Schematic of the experimental setup used for concentration of *E. coli* by ICP experiments. *Adapted from Perera et al. [98]

Perera et al. [98] fabricated a microfluidic paper-based analytical device (μPAD) (Figure 1.12). It consisted of a paper channel, a Nafion membrane and integrated microwire electrodes to supply the electric voltage. Then, the ICP effect was induced and negatively charged *E. coli* cells were pre-concentrated at the edge of the depletion zone of the anodic side of the Nafion membrane (ion-selective membrane). Finally, cell detection and counting were carried out by bacterial cell staining and fluorescence microscopy.

1.4.2.2 Microchip capillary electrophoresis (MCE)

Microchip capillary electrophoresis (MCE) is a family of electrokinetic separation methods that combines capillary electrophoresis and microchip technologies taking advantage of both techniques in terms of low sample consumption, automation and high-resolution separation. MCE devices usually consist of channels from 10 to 100 μm wide with a typical separation length between 3 and 10 cm that use electroosmotic flows (EOF) for the fluid manipulation.

MCE has been widely used in DNA analysis and has proved useful in bacteria separation [100,101]. Most MCE-based applications have used laser-induced fluorescence detection (LIF), but the technique can be combined with other methods such as PCR, absorbance, mass spectrometry, refractive index and Raman detection [102].

Zhang et al. [100] developed a system for the detection of *E. coli* that used bacteria-specific aptamers in conjunction with microchip capillary electrophoresis-coupled laser-induced fluorescence (MCE-LIF) (Figure 1.13). Fluorescent-labeled aptamers were incubated with 1 mL samples in the absence and presence of *E. coli* cells and injected into the MCE separation system. The separation of free aptamers and bacteria-aptamer complexes was achieved based on their different electrophoretic mobility. These differences in mobility stemmed from their charge to mass ratios. Finally, the detection was carried out by fluorescence.

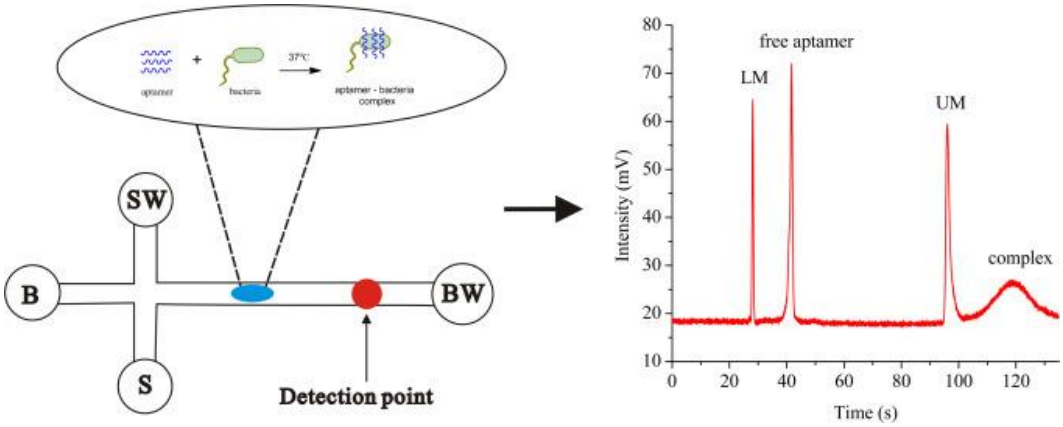

Figure 1.13. The principle of aptamer-based microchip capillary electrophoresis in bacterial detection. The electropherogram shows that bacteria-aptamer complex and free aptamer can be separated by MCE. The four reservoirs are shown as follows: Sample Reservoir (S); Sample Waste Reservoir (SW); Buffer Reservoir (B); Buffer Waste Reservoir (BW). Lower markers are displayed as LM, while upper markers are displayed as UM [100].

1.4.2.3 Dielectrophoretic field-flow fraction (DEP-FFF)

Dielectrophoresis (DEP) uses the effect of electrical polarization of particles under the influence of non-uniform electric fields to induce a translational motion. Thus, the particles are polarized forming an induced dipole. The positive and the negative extremes of the dipole are influenced by forces of different magnitude because of the non-uniformity of the electric field. These forces depend on the strength and frequency of the applied field, as well as on the conductivity of the supporting electrolyte. Thus, cells can be polarized in a highly conductive electrolyte suspension or diluted solutions and separated from other particles according to their dielectric properties [44].

However, due to some disadvantages as particle-particle interaction, DEP separation offers limited discrimination [22]. To solve this, different approaches have been developed. An example is the work of Kamuri et al., [103] combining DEP with field-flow fractionation (FFF) to separate *Escherichia coli* from *Saccharomyces cerevisiae* using a photonic detection system. Field-flow fractionation (FFF) is a family of methods that exploits the laminar hydrodynamic flow profile in a thin channel to drive the separation of different particle types based on their size. Thus, when DEP separation and FFF are combined a field perpendicular to the sample flow direction pumped through a long and narrow channel is applied, and microorganism are separated by different factors such as their diffusion or hydrodynamic and dielectric

characteristics. The assay was carried out with a mixed suspension of *E. coli* and *S. cerevisiae*, both at a concentration of 8 x 10^6 cell·mL^1 demonstrating the ability of the system to distinguish different cells without the need for recognition elements. However, the separation required 90 min and the ability of the system to work with lower cell densities was not proved.

1.4.2.4 Electrocoagulation (EC)

Electrocoagulation (EC) is an electrochemical process commonly used in wastewater treatment. In its simplest form, an electrocoagulation reactor consists of an electrolytic cell with a metallic anode and cathode. When connected to an external power source, the anode material is electrochemically corroded due to oxidation, while the cathode is subjected to passivation. Thus, the process uses electricity to dissolve metal present in the anode in order to produce hydroxides with adsorption capacity producing aggregates with colloids [104].

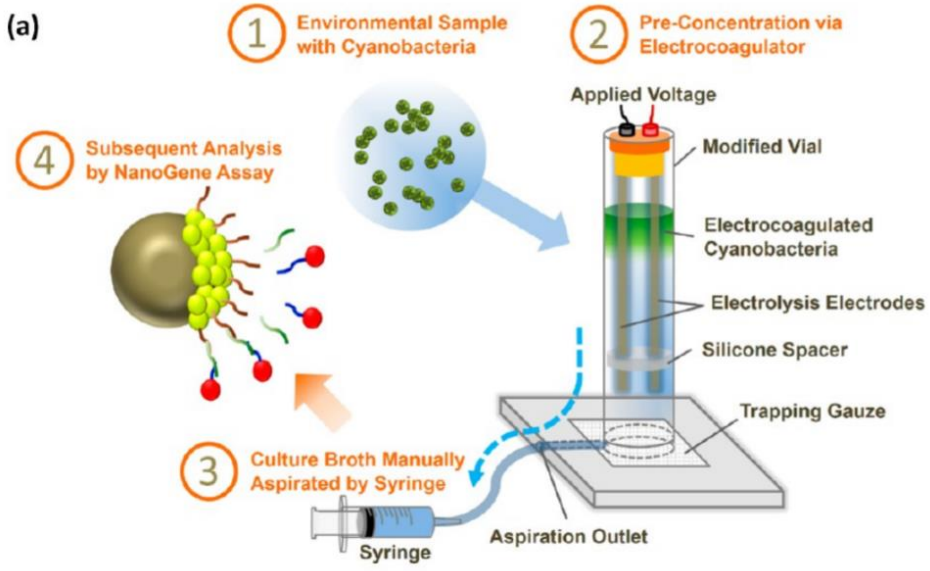

Figure 1.14. Schematic of the portable electrocoagulator for in situ cyanobacterial detection with NanoGene assay [105].

Although electrocoagulation has been typically used as a method for removing suspended matter, emulsions, dissolved contaminants and microorganisms, the technique has recently been adapted as a cyanobacteria concentration system prior

to detection [105]. A pocket-size portable electrocoagulator was developed consisting of aluminum anode and cathode electrodes. The hydration of aluminum hydroxide generated at the anode resulted in a variety of precipitates facilitating the coagulation of *Microcystis aeruginosa*. Finally, the electrocoagulator is coupled to the NanoGene assay suitable for in situ detection (Figure 1.14).

After 180 s of electrocoagulation of a 3 mL sample of *Microcystis*-containing river water, electrocoagulated cyanobacteria were trapped on a woven cloth to be subjected to DNA extraction by the NanoGene assay. Preconcentration by electrocoagulation showed a similar performance to a conventional centrifuge with a concentration efficiency of 60%. However, cell densities lower than 10^4 cell·mL^{-1} could not be concentrated. Thus, the system showed the ability to concentrate cyanobacterial cells in real samples but at concentrations already at bloom stages.

Table 1.3. Resume of some experimental parameters of systems using electric separation.

Pre-treatment		Bacteria	Vol. (mL)	Bioreceptor	Transductor	LoD (CFU·mL^{-1})	Assay time (min)		Integration	Portability	Reference
							Con.	Detec.			
Electric separation	MCE	*E. coli*	1	Aptamer	Fluorescence	3.7 10^2	-		×	×	[100]
	ICP	*E. coli*	-	-	Fluorescence	10^3 – 10^4	-		×	×	[98]
	DE	*E.coli* *S. cerevisae*	-	-	Light scattering	-	90		×	×	[103]
	EC	*Microcystis* *P. aeruginosa*	3	-	-	>10^4	0.3	-	×	✓	[106]

1.4.3 Centrifugal microfluidics

Centrifugal microfluidic devices use automated rotational motors to manipulate fluid flow through channels using centrifugal forces [107], which depend on rotation rates, geometry, and location of channels and reservoirs, besides fluid properties. Thus, using different spinning profiles, these systems are able to integrate processes such as separation, mixing, reaction and detection [108].

Zhang et al., [107] presented an integrated centrifugal microfluidic device able to sample pre-concentration, filtration, incubation and target capture, and detection (Figure 1.15). First, a 50 µL sample of water was added to the device to pre-concentrate bacteria by sedimentation using high rotational speeds. The supernatant was removed by deceleration. The pre-concentrated sample was combined with gold nanoparticles conjugated with anti-*E.coli* antibodies and analyzed by lateral flow immunoassay. The limit of detection of the device was 10^5 CFU·mL^1, 10 times lower than the LoD for a non-concentrated sample. However, although the system showed the capacity for sample concentration, the processed volume was small and the obtained limit of detection unsatisfactory.

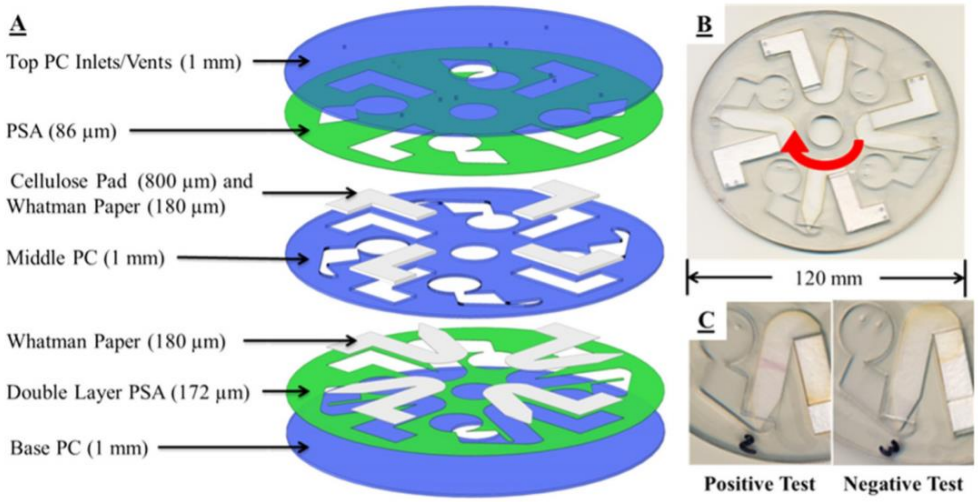

Figure 1.15. (A) The constructed paper-polymer hybrid device consists of polycarbonate (PC), pressure-sensitive adhesive (PSA) and paper inserts. (B) A completed device is shown with the direction of rotation indicated by a red arrow. (C) The paper inset is a colorimetric lateral flow immunoassay with a positive and a negative test result for pathogenic *E. coli* shown in the figure [107].

Centrifuge based systems can also be combined with superabsorbent polymer (SAP) microspheres [109]. SAP microspheres contained itaconic acid, which drove the absorption of water through osmosis, retaining water up to 1000 times their initial dry weight. Additionally, the absorption of microorganisms on their surface is minimized due to the electrostatic forces conferred by the negative charges. The system consisted of a centrifuge tube where 40 mL *E. coli* sample was added and kept in the upper chamber in contact with the SAP microspheres (Figure 1.16). After 15 min almost all the liquid of the sample was absorbed, and the residual volume (4mL) containing bacteria was transferred to the lower chamber by manual

centrifugation (500 rpm) to perform the detection measurements by RT-PCR. Thus, the method allowed increasing bacteria concentration by 10 folds with an efficiency of 87% in only 20 min. Therefore, a low cost, portable and easy to use concentration system was obtained for rapid on-site microbial analysis. However, this concentration system should be integrated to a detection system to get a complete on-site bacterial detection device. Moreover, the system was tested for bacterial cell concentrations higher than 10^4 CFU·mL^{-1}, so the ability of the method to work with lower bacterial quantities should be proved.

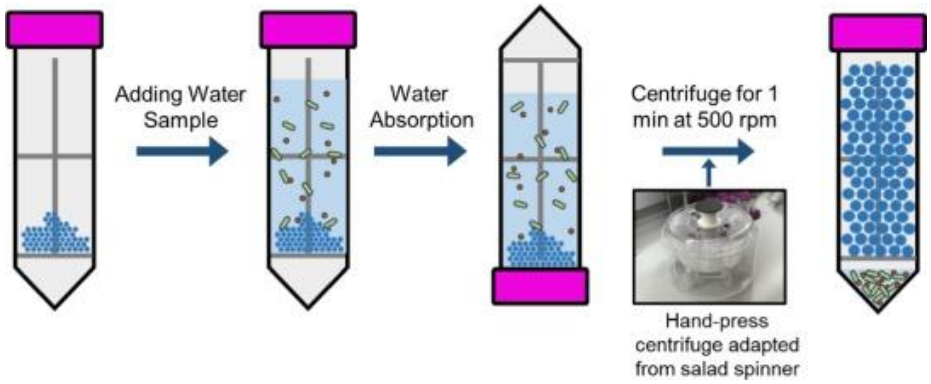

Figure 1.16. The tube system designed for microbial pathogen concentration using SAP microspheres. The tube is composed of SAP microspheres and a 3D-printed filter. After adding the water sample, the tube is left to stand for 20 min for the full absorption of water by SAP. Non-absorbed water is pushed to the lower chamber using a hand-press centrifuge [109].

Table 4. Resume of some experimental parameters of systems using centrifugation.

Pre-treatment	**Bacteria**	**Vol. (mL)**	**Bioreceptor**	**Transductor**	**LoD (CFU·mL^{-1})**	**Assay time (min)**		**Integration**	**Portability**	**Ref**
						Con.	Detec.			
Centrifugation	*E. coli*	0.05	Antibody	Absorbance/ Visual evaluation	10^5	15	60	✓	✓	[107]
	E.coli	40	-	-	-	20	-	×	✓	[109]

1.4.4 Membrane-based separation

Membrane filtration is an easy, direct and straightforward way to reduce large sample volumes and concentrate the target of interest. This technique involves the use of porous membranes that act as selective barriers by size exclusion [110]. Among membranes, microfiltration membranes retain particles in size range of 0.1–10 µm in diameter and are available in materials such as ceramic, steel, and fibrous materials [111]. The main advantage offered by this concentration method is the possibility to work with large sample volumes increasing the sensitivity of the system and complying with the requirements established in the legislation for the analysis of the environmental samples. However, the most challenging part of these systems is their miniaturization and integration into automated sensing devices [88].

Zhang et al.,[112] proposed an automated bacterial concentration and recovery system (ABCRS) (Figure 1.17) based on the combination of a ceramic membrane with a tangential flow filtration technique. The system was able to concentrate a large amount of water (1-2 liters) to a few milliliters (≈ 5 mL) carrying out several cycles of concentration and recovery by backflushing through the membrane. In less than an hour high recovery efficiencies (90%) could be achieved. The objective was to obtain a concentrated sample volume adaptable to the volumetric capacities of the biosensors later used for bacterial detection. So, it may be coupled to a suitable detection system.

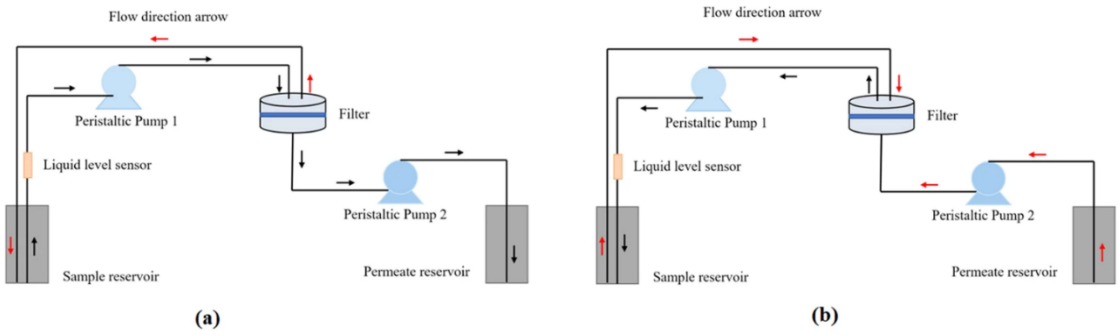

Figure 1.17. Schematic diagrams of an automated bacterial concentration and recovery system (ABCRS) with the tangential flow filtration technique used in the pre-enrichment process for (**a**) forward, and (**b**) backward flow procedure [112].

On the other hand Martin et al.,[113] proposed the use of a pre-concentration step by filtering 1 L samples through 0.22 µm pore-size membranes. This pre-

concentration step allowed them to improve the detection limit of their amperometric magnetoimmunoassay from 10^4 CFUs·mL^{-1} of *Legionella* to 10 CFU·mL^{-1}. Nevertheless, the concentration step was not integrated into the detection system as cells needed to be eluted by vortexing and concentrated for a second time by centrifugation to finally obtain 500 µL of concentrated bacteria solution (x2000).

Sensors offer the possibility of obtaining simpler, faster and more portable detection systems than conventional methods. However, the low concentrations at which the pathogens are found in environmental waters make the integration of concentration systems to detection devices a must. Membrane-based concentration methods seem to be currently the only capable of handling volumes from milliliter to liters. However, its integration into the detection systems to obtain a single concentration-detection system remains challenging.

Table 1.5. Resume of some experimental parameters of systems using membrane filtration

Pre-treatment	**Bacteria**	**Vol. (mL)**	**Bioreceptor**	**Transductor**	**LoD (CFU·mL^{-1})**	**Assay time (min)**		**Integration**	**Portability**	**Ref**
						Con.	Detec.			
Membrane filtration	*Legionella*	1 L	Antibody	Amperometry	10 CFU·mL^{-1}	3 h	×	×	×	[113]
	E. coli	1 L	-	-	-	1h	×	×	×	[112]

1.5 AIM AND OBJECTIVES

The main goal of this Ph.D. thesis is the development of integrated devices for waterborne pathogen concentration and detection. The greatest hindrance when detecting waterborne bacteria is the sample volume required to achieve low detection limits established by the regulatory frameworks. The aim of the work described in this dissertation is the design of concentration-detection integrated systems taking advantage of filter-based concentration processes coupled to detection systems that suit well with the target bacteria.

In order to fulfill this aim, the following specific objectives have been carried out:

1. **Development of an on-filter protocol for the concentration and detection of microorganisms present in water samples**. For this, an immunoassay protocol has to be developed using *E. coli* and *Legionella* as model microorganisms and filtration membranes as the reaction support. The results of this work are presented in Chapter 2 and 3: "On-filter protocol for concentration and detection *Escherichia coli*" and "On-filter protocol for concentration and detection of *Legionella pneumophila*"
2. **Optimization of an amperometric transduction method for bacteria detection.** Design and fabrication of screen-printed electrodes and characterization of the amperometric measurements of the redox substrate employed in the immunoassay protocol for *Legionella* detection. The results of this work are presented in Chapter 4: "On-filter immunoassay and amperometric measurements for rapid detection of *Legionella pnemumophila*"
3. **Adaptation of the concentration platform for on-filter concentration and detection of cyanobacteria.** Modification of the designed sensing device for carrying out a fluorescence detection by the integration of fiber-optics, and optimization of the measurements using *Synechocystis* as model cyanobacteria. The results of this work are presented in Chapter 5: "Development of a photonic device for early warning of cyanobacterial blooms"
4. **Design of Integrated prototype for on-site concentration and detection bacteria.** Fabrication of a portable photonic prototype provided with fluidic and miniaturized optical components for the on-site detection of bacteria. The

results are presented in Chapter 6: “Photonic prototype for the on-site concentration and detection of bacteria”.

REFERENCES:

[1] F. Ramírez-Castillo, A. Loera-Muro, M. Jacques, P. Garneau, F. Avelar-González, J. Harel, A. Guerrero-Barrera, Waterborne Pathogens: Detection Methods and Challenges, Pathogens. 4 (2015) 307–334. https://doi.org/10.3390/pathogens4020307.

[2] C.P. Gerba, I.L. Pepper, Microbial Contaminants, Environ. Pollut. Sci. (2019) 191–217. https://doi.org/10.1016/B978-0-12-814719-1.00013-6.

[3] R.A. Deshmukh, K. Joshi, S. Bhand, U. Roy, Recent developments in detection and enumeration of waterborne bacteria: a retrospective minireview, Microbiologyopen. 5 (2016) 901–922. https://doi.org/10.1002/mbo3.383.

[4] P.K. Pandey, P.H. Kass, M.L. Soupir, S. Biswas, V.P. Singh, Contamination of water resources by pathogenic bacteria, AMB Express. 4 (2014) 1–16. https://doi.org/10.1186/s13568-014-0051-x.

[5] WHO, WHO global water, sanitation and hygiene, 2018. https://www.who.int/water_sanitation_health/publications/global-water-sanitation-and-hygiene-annual-report-2018/en/ (accessed October 22, 2019).

[6] WHO, Waterborne disease - From MDGS to SDGs (2015), 2015. https://www.who.int/gho/publications/mdgs-sdgs/MDGs-SDGs2015_chapter5_snapshot_waterborne_diseases.pdf?ua=1 (accessed October 23, 2019).

[7] P. Payment, Health effects of water consumption and water quality, in: Handb. Water Wastewater Microbiol., Academic Press, 2003: pp. 209–219. https://doi.org/10.1016/B978-012470100-7/50014-5.

[8] J.K. Griffiths, Waterborne Diseases, Int. Encycl. Public Heal. (2017) 388–401. https://doi.org/10.1016/B978-0-12-803678-5.00490-2.

[9] B. Højris, S.C.B. Christensen, H.J. Albrechtsen, C. Smith, M. Dahlqvist, A novel, optical, on-line bacteria sensor for monitoring drinking water quality, Sci. Rep. 6 (2016) 23935. https://doi.org/10.1038/srep23935.

[10] F.J. Ferrero Martín, M. Valledor Llopis, J.C. Campo Rodriguez, L. Marín Fernández, I. Gutiérrez-Del-Río Menéndez, J. Fernández Fernández, F. Lombó

Brugos, N. Cobián Fernandez, F. Olmos Fernández Corugedo, I. Méndez Suarez, A Novel Handheld Fluorimeter for Rapid Detection of Escherichia coli in Drinking Water, IEEE Sens. J. 16 (2016) 5136–5144. https://doi.org/10.1109/JSEN.2016.2558649.

[11] C. Wang, F. Madiyar, C. Yu, J. Li, Detection of extremely low concentration waterborne pathogen using a multiplexing self-referencing SERS microfluidic biosensor, J. Biol. Eng. 11 (2017) 9. https://doi.org/10.1186/s13036-017-0051-x.

[12] M. José Figueras, J.J. Borrego, New perspectives in monitoring drinking water microbial quality, Int. J. Environ. Res. Public Health. 7 (2010) 4179–4202. https://doi.org/10.3390/ijerph7124179.

[13] Y.A. Pachepsky, A. Allende, L. Boithias, K. Cho, R. Jamieson, N. Hofstra, M. Molina, Microbial water quality: Monitoring and modeling, J. Environ. Qual. 47 (2018) 931–938. https://doi.org/10.2134/jeq2018.07.0277.

[14] O. of W. US EPA, 5.11 Fecal Bacteria, (n.d.).

[15] E. Schroeder, S. Wuertz, Bacteria, Handb. Water Wastewater Microbiol. (2003) 57–68. https://doi.org/10.1016/B978-012470100-7/50004-2.

[16] Word Health Organization (WHO), WHO | Guidelines for drinking-water quality: fourth edition incroporating the first addenum, 4th editio, Geneva, 2017. http://www.who.int (accessed November 21, 2019).

[17] C.J. Hurst, R.L. Crawford, J.L. Garland, D.A. Lipson, A.L. Mills, L.D. Stetzenbach, Manual of environmental microbiology (3rd Edition), Third edit, American Society of Microbiology (ASM), 2007. https://doi.org/10.1128/9781555815882.

[18] G. Bitton, Wastewater Microbiology: Fourth Edition, John Wiley and Sons, 2010. https://doi.org/10.1002/9780470901243.

[19] H. Leclerc, Relationships between common bacteria and pathogens in drinking-water, in: J. Bartram, J. Cotruvo, M. Exner, C. Fricker, A. Glasmacher (Eds.), Heterotrophic Plate Counts Ans Drink. Saf., IWA Publishing, London, UK, 2003: pp. 80–118.

[20] P. Lebaron, B. Cournoyer, K. Lemarchand, S. Nazaret, P. Servais, Environmental and human pathogenic microorganisms, in: Environ. Microbiol.

Fundam. Appl., Springer Netherlands, 2015: pp. 619–658. https://doi.org/10.1007/978-94-017-9118-2_15.

[21] World Health Organization, Guidelines for safe recreational water environments: Volume 2. Swimming pools and similar environments, World Heal. Organ. 2 (2006) 146.

[22] P.R.C. Gascoyne, J. Vykoukal, Particle separation by dielectrophoresis, Electrophoresis. 23 (2002) 1973. https://doi.org/10.1002/1522-2683(200207)23:13<1973::AID-ELPS1973>3.0.CO;2-1.

[23] H. Bridle, K. Gilbride, J. Green, Existing Methods of Waterborne Pathogen Detection, in: Waterborne Pathog. Detect. Methods Appl., Academic Press, 2013: pp. 41–64. https://doi.org/10.1016/B978-0-444-59543-0.00003-7.

[24] G. Saxena, R.N. Bharagava, G. Kaithwas, A. Raj, Microbial indicators, pathogens and methods for their monitoring in water environment, J. Water Health. 13 (2015) 319–339. https://doi.org/10.2166/wh.2014.275.

[25] J. Wu, S.C. Long, D. Das, S.M. Dorner, Are microbial indicators and pathogens correlated? A statistical analysis of 40 years of research, J. Water Health. 9 (2011) 265–278. https://doi.org/10.2166/wh.2011.117.

[26] S. Ishii, M.J. Sadowsky, Escherichia coli in the environment: Implications for water quality and human health, Microbes Environ. 23 (2008) 101–108. https://doi.org/10.1264/jsme2.23.101.

[27] J.B. Kaper, J.P. Nataro, Diarrheagenic Escherichia coli Strains, Clin. Microbiol. Rev. 11 (1998) 142–201. https://doi.org/10.1128/CMR.11.1.142.

[28] H. Leclerc, L. Schwartzbrod, E. Dei-Cas, Microbial agents associated with waterborne diseases, Crit. Rev. Microbiol. 28 (2002) 371–409. https://doi.org/10.1080/1040-840291046768.

[29] S.L. Percival, D.W. Williams, Escherichia coli, in: Microbiol. Waterborne Dis. Microbiol. Asp. Risks Second Ed., Elsevier, 2013: pp. 89–117. https://doi.org/10.1016/B978-0-12-415846-7.00006-8.

[30] Health Canada, Guidance on waterborne bacterial pathogens, 2013. www.healthcanada.gc.ca (accessed October 24, 2019).

[31] C. Caicedo, K.H. Rosenwinkel, M. Exner, W. Verstraete, R. Suchenwirth, P.

Hartemann, R. Nogueira, Legionella occurrence in municipal and industrial wastewater treatment plants and risks of reclaimed wastewater reuse: Review, Water Res. 149 (2019) 21–34. https://doi.org/10.1016/j.watres.2018.10.080.

[32] H. Tronel, P. Hartemann, Overview of diagnostic and detection methods for legionellosis and Legionella spp., Lett. Appl. Microbiol. 48 (2009) 653–656. https://doi.org/10.1111/j.1472-765X.2009.02570.x.

[33] E. Jubete, O.A. Loaiza, E. Ochoteco, J.A. Pomposo, H. Grande, J. Rodríguez, Nanotechnology: A Tool for Improved Performance on Electrochemical Screen-Printed (Bio)Sensors, J. Sensors. 2009 (2009) 1–13. https://doi.org/10.1155/2009/842575.

[34] A. Sikora, M. Wójtowicz-Bobin, M. Kozioł-Montewka, A. Magryś, I. Gładysz, Prevalence of Legionella pneumophila in water distribution systems in hospitals and public buildings of the Lublin region of eastern Poland, Ann. Agric. Environ. Med. 22 (2015) 195–201. https://doi.org/10.5604/12321966.1152064.

[35] K.M.E. Emde, H. Mao, G.R. Finch, Detection and occurrence of waterborne bacterial and viral pathogens, Water Environ. Res. 64 (1992) 641–647. https://doi.org/10.1002/j.1554-7531.1992.tb00046.x.

[36] ECDC, Legionnaires' disease Annual epidemiological report for 2017, Eur. Cent. Dis. Prev. Control. (2019) 7. https://doi.org/10.1007/s00339-004-2615-0.

[37] World Health Organization, Legionella and the Prevention of Legionellosis, 2007. https://www.who.int/water_sanitation_health/emerging/legionella.pdf (accessed September 4, 2019).

[38] Real Decreto 865/2003, de 4 de julio, por el que se establecen los criterios higiénico-sanitariois para la prevención y control de la legionelosis. BOE, Boletín Oficial del Estado, 2003.

[39] World Health Organization (WHO 2003), Chapter 8: Algae and cyanobacteria in fresh water, in: Guidel. Safe Recreat. Waters, Vol. 1 – Coast. Fresh Waters, 1st ed., WHO Publishing, Geneva, 2003: pp. 136–158. https://www.who.int/water_sanitation_health/bathing/srwe1-chap8.pdf (accessed May 16, 2019).

[40] Environmental Protection Agency, Cyanobacteria and Cyanotoxins: Information for Drinking Water Systems, United States Environ. Prot. Agency. (2014) 1–9. https://www.epa.gov/sites/production/files/2014-08/documents/cyanobacteria_factsheet.pdf (accessed March 19, 2018).

[41] B. Puschner, C. Moore, Cyanobacteria, in: Small Anim. Toxicol. Third Ed., Elsevier Inc., 2012: pp. 533–540. https://doi.org/10.1016/B978-1-4557-0717-1.00043-0.

[42] W.F. Vincent, Protists, Bacteria and Fungi: Planktonic and Attached - Cyanobacteria, in: G.E. Likens (Ed.), Encycl. Inl. Waters, Elsevier, 2009: pp. 226–232. https://doi.org/10.1016/B978-012370626-3.00127-7.

[43] J. Chorus, Ingrid & Bartram, Toxic cyanobacteria in water. A guide to their public health consequences, monitoring, and management / edited by Ingrid Chorus and Jamie Bertram., World Heal. Organ. (1999). http://www.earthprint.com (accessed August 26, 2019).

[44] Ó. Castillo-Fernandez, N. Uria, F.X. Muñoz, A. Bratov, Cell Concentration Systems for Enhanced Biosensor Sensitivity, in: Biosens. - Micro Nanoscale Appl., InTech, 2015. https://doi.org/10.5772/61088.

[45] H.W. Paerl, R.S. Fulton, P.H. Moisander, J. Dyble, Harmful freshwater algal blooms, with an emphasis on cyanobacteria., ScientificWorldJournal. 1 (2001) 76–113. https://doi.org/10.1100/tsw.2001.16.

[46] G.R. Shaw, D.P. Moore, C. Garnett, Eutrophication and Algal Blooms, Encycl. Life Support Syst. 2 (2003) 1–21. http://www.eolss.net/Sample-Chapters/C06/E6-13-04-04.pdf (accessed April 3, 2018).

[47] K.G. Sellner, G.J. Doucette, G.J. Kirkpatrick, Harmful algal blooms: Causes, impacts and detection, J. Ind. Microbiol. Biotechnol. 30 (2003) 383–406. https://doi.org/10.1007/s10295-003-0074-9.

[48] A. Sinden, S.C. Sinang, Cyanobacteria in aquaculture systems: linking the occurrence, abundance and toxicity with rising temperatures, Int. J. Environ. Sci. Technol. 13 (2016) 2855–2862. https://doi.org/10.1007/s13762-016-1112-2.

[49] F. Choo, A. Zamyadi, K. Newton, G. Newcombe, L. Bowling, R. Stuetz, R.K. Henderson, Performance evaluation of in situ fluorometers for real-time

cyanobacterial monitoring, H2Open J. 1 (2018) 26–46. https://doi.org/10.2166/h2oj.2018.009.

[50] B. K. Dwivedi, Potential Risk Assessment of Cyanotoxin for Water Resources, Int. J. Curr. Microbiol. Appl. Sci. 7 (2018) 3604–3616. https://doi.org/10.20546/ijcmas.2018.702.429.

[51] Y. Blanco, A. Quesada, I. Gallardo-Carreño, J. Aguirre, V. Parro, CYANOCHIP: An antibody microarray for high-taxonomical-resolution cyanobacterial monitoring, Environ. Sci. Technol. 49 (2015) 1611–1620. https://doi.org/10.1021/es5051106.

[52] J.M. O'Neil, T.W. Davis, M.A. Burford, C.J. Gobler, The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change, Harmful Algae. 14 (2012) 313–334. https://doi.org/10.1016/j.hal.2011.10.027.

[53] W. Teng, Z. Wu, J. Fan, H. Chen, D. Feng, Y. Lv, J. Wang, A.M. Asiri, D. Zhao, Ordered mesoporous carbons and their corresponding column for highly efficient removal of microcystin-LR, Energy Environ. Sci. 6 (2013) 2765–2776. https://doi.org/10.1039/c3ee41775a.

[54] J.-T. Kuo, C.-Y. Cheng, H.-H. Huang, C.-F. Tsao, Y.-C. Chung, A rapid method for the detection of representative coliforms in water samples: polymerase chain reaction-enzyme-linked immunosorbent assay (PCR-ELISA), J. Ind. Microbiol. {&} Biotechnol. 37 (2010) 237–244. https://doi.org/10.1007/s10295-009-0666-0.

[55] A. Rompré, P. Servais, J. Baudart, M.-R. De-Roubin, P. Laurent, Detection and enumeration of coliforms in drinking water: current methods and emerging approaches, J. Microbiol. Methods. 49 (2002) 31–54. https://doi.org/10.1016/S0167-7012(01)00351-7.

[56] O. Lazcka, F.J. Del Campo, F.X. Muñoz, Pathogen detection: A perspective of traditional methods and biosensors, Biosens. Bioelectron. 22 (2007) 1205–1217. https://doi.org/10.1016/j.bios.2006.06.036.

[57] J. Shah, S. Chemburu, E. Wilkins, I. Abdel-Hamid, Rapid Amperometric Immunoassay for Escherichia coli Based on Graphite Coated Nylon Membranes, Electroanalysis. 15 (2003) 1809–1814. https://doi.org/10.1002/elan.200302791.

[58] D.R. Thvenot, K. Toth, R.A. Durst, G.S. Wilson, Electrochemical biosensors: Recommended definitions and classification (Technical Report), Pure Appl. Chem. 71 (1999) 2333–2348. https://doi.org/10.1351/pac199971122333.

[59] K. Cammann, U. Lemke, A. Rohen, J. Sander, H. Wilken, B. Winter, Chemical Sensors and Biosensors—Principles and Applications, Angew. Chemie Int. Ed. English. 30 (1991) 516–539. https://doi.org/10.1002/anie.199105161.

[60] B. Bozal-Palabiyik, A. Gumustas, S.A. Ozkan, B. Uslu, Biosensor-Based Methods for the Determination of Foodborne Pathogens, in: Foodborne Dis., Academic Press, 2018: pp. 379–420. https://doi.org/10.1016/B978-0-12-811444-5.00012-9.

[61] C.I.L. Justino, A.C. Freitas, R. Pereira, A.C. Duarte, T.A.P. Rocha Santos, Recent developments in recognition elements for chemical sensors and biosensors, TrAC - Trends Anal. Chem. 68 (2015) 2–17. https://doi.org/10.1016/j.trac.2015.03.006.

[62] M.A. Morales, J.M. Halpern, Guide to Selecting a Biorecognition Element for Biosensors, Bioconjug. Chem. 29 (2018) 3231–3239. https://doi.org/10.1021/acs.bioconjchem.8b00592.

[63] H. Bridle, M. Desmulliez, Biosensors for the Detection of Waterborne Pathogens, in: Waterborne Pathog. Detect. Methods Appl., Academic Press, 2013: pp. 189–229. https://doi.org/10.1016/B978-0-444-59543-0.00002-5.

[64] S.P. Masaaki K, Rapid Detection Technologies for Monitoring Microorganisms in Water, Biosens. J. 03 (2014). https://doi.org/10.4172/2090-4967.1000109.

[65] S. Kumar, M. Nehra, J. Mehta, N. Dilbaghi, G. Marrazza, A. Kaushik, Point-of-care strategies for detection of waterborne pathogens, Sensors (Switzerland). 19 (2019). https://doi.org/10.3390/s19204476.

[66] A. Davydova, M. Vorobjeva, D. Pyshnyi, S. Altman, V. Vlassov, A. Venyaminova, Aptamers against pathogenic microorganisms, Crit. Rev. Microbiol. 42 (2016) 847–865. https://doi.org/10.3109/1040841X.2015.1070115.

[67] M. Hoyos-Nogués, F.J. Gil, C. Mas-Moruno, Antimicrobial peptides: Powerful biorecognition elements to detect bacteria in biosensing technologies, Molecules. 23 (2018) 1–24. https://doi.org/10.3390/molecules23071683.

[68] A.A. Bahar, D. Ren, Antimicrobial peptides, Pharmaceuticals. 6 (2013) 1543–1575. https://doi.org/10.3390/ph6121543.

[69] D. Wang, T. Hinkley, J. Chen, J.N. Talbert, S.R. Nugen, Phage based electrochemical detection of: Escherichia coli in drinking water using affinity reporter probes, Analyst. 144 (2019) 1345–1352. https://doi.org/10.1039/c8an01850b.

[70] Z. Wang, D. Wang, A.J. Kinchla, D.A. Sela, S.R. Nugen, Rapid screening of waterborne pathogens using phage-mediated separation coupled with real-time PCR detection, Anal. Bioanal. Chem. 408 (2016) 4169–4178. https://doi.org/10.1007/s00216-016-9511-2.

[71] A. Rasooly, K.E. Herold, Biosensors for the analysis of food- and waterborne pathogens and their toxins., J. AOAC Int. 89 (n.d.) 873–83. http://www.ncbi.nlm.nih.gov/pubmed/16792089 (accessed January 21, 2020).

[72] N. Kumar, Y. Hu, S. Singh, B. Mizaikoff, Emerging biosensor platforms for the assessment of water-borne pathogens, Analyst. 143 (2018) 359–373. https://doi.org/10.1039/C7AN00983F.

[73] A.A. Zuber, E. Klantsataya, A. Bachhuka, Biosensing, in: Compr. Nanosci. Nanotechnol., Elsevier, 2019: pp. 105–126. https://doi.org/10.1016/b978-0-12-803581-8.10410-2.

[74] A. Hulanicki, S. Glab, F. Ingman, Chemical sensors definitions and classification, Pure Appl. Chem. 63 (1991) 1247–1250. https://doi.org/10.1351/pac199163091247.

[75] X. Wang, O.S. Wolfbeis, Fiber-Optic Chemical Sensors and Biosensors (2015–2019), Anal. Chem. (2019) acs.analchem.9b04708. https://doi.org/10.1021/acs.analchem.9b04708.

[76] G.-P. Nikoleli, D.P. Nikolelis, C.G. Siontorou, S. Karapetis, T. Varzakas, Novel Biosensors for the Rapid Detection of Toxicants in Foods, in: 2018: pp. 57–102. https://doi.org/10.1016/bs.afnr.2018.01.003.

[77] V. Velusamy, K. Arshak, O. Korostynska, K. Oliwa, C. Adley, An overview of foodborne pathogen detection: In the perspective of biosensors, Biotechnol. Adv. 28 (2010) 232–254. https://doi.org/10.1016/j.biotechadv.2009.12.004.

[78] N. Shanker, S.L. Bane, Basic Aspects of Absorption and Fluorescence Spectroscopy and Resonance Energy Transfer Methods, Methods Cell Biol. 84 (2008) 213–242. https://doi.org/10.1016/S0091-679X(07)84008-8.

[79] H. Itagaki, Fluorescence Spectroscopy, in: Exp. Methods Polym. Sci. Mod. Methods Polym. Res. Technol., Elsevier Inc., 2012: pp. 155–260. https://doi.org/10.1016/B978-0-08-050612-8.50009-X.

[80] F. Shakibaie, L. Lamard, H. Rubinsztein-Dunlop, L.J. Walsh, Application of Fluorescence Spectroscopy for Microbial Detection to Enhance Clinical Investigations, in: Phot. Count. - Fundam. Appl., InTech, 2018. https://doi.org/10.5772/intechopen.73616.

[81] M.T.Z. Johnson, I & Spencer, Fluorescence Fundamentals | Thermo Fisher Scientific, (2016) 1–9.

[82] Y. Chen, Z. Wang, Y. Liu, X. Wang, Y. Li, P. Ma, B. Gu, H. Li, Recent advances in rapid pathogen detection method based on biosensors, Eur. J. Clin. Microbiol. Infect. Dis. 37 (2018) 1021–1037. https://doi.org/10.1007/s10096-018-3230-x.

[83] D. Grieshaber, R. MacKenzie, J. Vörös, E. Reimhult, Electrochemical Biosensors - Sensor Principles and Architectures, Sensors. 8 (2008) 1400–1458. https://doi.org/10.3390/s80314000.

[84] R. Antiochia, G. Favero, M.E. Conti, C. Tortolini, Affinity-based biosensors for pathogenic bacteria detection, Int. J. Environ. Technol. Manag. 18 (2015) 185. https://doi.org/10.1504/IJETM.2015.071140.

[85] A. Ahmed, J. V. Rushworth, N.A. Hirst, P.A. Millner, Biosensors for whole-cell bacterial detection, Clin. Microbiol. Rev. 27 (2014) 631–646. https://doi.org/10.1128/CMR.00120-13.

[86] K.C. Honeychurch, Printed thick-film biosensors, in: Print. Film. Mater. Sci. Appl. Sensors, Electron. Photonics, Elsevier, 2012: pp. 366–409. https://doi.org/10.1533/9780857096210.2.366.

[87] U. Dharmasiri, M.A. Witek, A.A. Adams, S.A. Soper, Microsystems for the capture of low-abundance cells., Annu. Rev. Anal. Chem. 3 (2010) 409–31. https://doi.org/10.1146/annurev.anchem.111808.073610.

[88] J.T. Connelly, A.J. Baeumner, Biosensors for the detection of waterborne

pathogens, Anal. Bioanal. Chem. 402 (2012) 117–127. https://doi.org/10.1007/s00216-011-5407-3.

[89] L. Xue, L. Zheng, H. Zhang, X. Jin, J. Lin, An ultrasensitive fluorescent biosensor using high gradient magnetic separation and quantum dots for fast detection of foodborne pathogenic bacteria, Sensors Actuators, B Chem. 265 (2018) 318–325. https://doi.org/10.1016/j.snb.2018.03.014.

[90] J. Porter, R. Pickup, Magnetic particle–based separation techniques for monitoring bacteria from natural environments, Methods Biotechnol. 12 (2015) 75–96. https://doi.org/10.1385/0-89603-566-2:75.

[91] K.Y. Castillo-Torres, E.S. McLamore, D.P. Arnold, A high-throughput microfluidic magnetic separation (μFMS) platform for water quality monitoring, Micromachines. 11 (2020) 16. https://doi.org/10.3390/mi11010016.

[92] Y. Zhang, C. Yan, H. Yang, J. Yu, H. Wei, Rapid and selective detection of E. coli O157:H7 combining phagomagnetic separation with enzymatic colorimetry, Food Chem. 234 (2017) 332–338. https://doi.org/10.1016/j.foodchem.2017.05.013.

[93] J. Shi, M. Wang, Z. Sun, Y. Liu, J. Guo, H. Mao, F. Yan, Aggregation-induced emission-based ionic liquids for bacterial killing, imaging, cell labeling, and bacterial detection in blood cells, Acta Biomater. 97 (2019) 247–259. https://doi.org/10.1016/j.actbio.2019.07.039.

[94] S.A. Hice, K.D. Clark, J.L. Anderson, B.F. Brehm-Stecher, Capture, Concentration, and Detection of Salmonella in Foods Using Magnetic Ionic Liquids and Recombinase Polymerase Amplification, Anal. Chem. 91 (2019) 1113–1120. https://doi.org/10.1021/acs.analchem.8b04751.

[95] K.D. Clark, O. Nacham, J.A. Purslow, S.A. Pierson, J.L. Anderson, Magnetic ionic liquids in analytical chemistry: A review, Anal. Chim. Acta. 934 (2016) 9–21. https://doi.org/10.1016/j.aca.2016.06.011.

[96] E. Santos, J. Albo, A. Irabien, Magnetic ionic liquids: Synthesis, properties and applications, RSC Adv. 4 (2014) 40008–40018. https://doi.org/10.1039/c4ra05156d.

[97] K.D. Clark, J.A. Purslow, S.A. Pierson, O. Nacham, J.L. Anderson, Rapid preconcentration of viable bacteria using magnetic ionic liquids for PCR

amplification and culture-based diagnostics, Anal. Bioanal. Chem. 409 (2017) 4983–4991. https://doi.org/10.1007/s00216-017-0439-y.

[98] A.T.K. Perera, S. Pudasaini, S.S.U. Ahmed, D.T. Phan, Y. Liu, C. Yang, Rapid pre-concentration of Escherichia coli in a microfluidic paper-based device using ion concentration polarization, Electrophoresis. (2019) elps.201900303. https://doi.org/10.1002/elps.201900303.

[99] D.T. Phan, S.A.M. Shaegh, C. Yang, N.T. Nguyen, Sample concentration in a microfluidic paper-based analytical device using ion concentration polarization, Sensors Actuators, B Chem. 222 (2016) 735–740. https://doi.org/10.1016/j.snb.2015.08.127.

[100] Y. Zhang, L. Zhu, P. He, F. Zi, X. Hu, Q. Wang, Sensitive assay of Escherichia coli in food samples by microchip capillary electrophoresis based on specific aptamer binding strategy, Talanta. 197 (2019) 284–290. https://doi.org/10.1016/j.talanta.2019.01.040.

[101] C.S. Henry, Microchip capillary electrophoresis: an introduction., in: Methods Mol. Biol., 2006: pp. 1–10. https://doi.org/10.1385/1-59745-076-6:1.

[102] N.A. Lacher, K.E. Garrison, R.S. Martin, S.M. Lunte, Microchip capillary electrophoresis/ electrochemistry, Electrophoresis. 22 (2001) 2526–2536. https://doi.org/10.1002/1522-2683(200107)22:12<2526::AID-ELPS2526>3.0.CO;2-K.

[103] M.F. Kamuri, Z.Z. Abidin, M.H. Yaacob, M.N. Hamidon, N.A.M. Yunus, S. Kamarudin, Separation and detection of Escherichia coli and saccharomyces cerevisiae using a microfluidic device integrated with an optical fibre, Biosensors. 9 (2019). https://doi.org/10.3390/bios9010040.

[104] N. Boudjema, N. Drouiche, N. Abdi, H. Grib, H. Lounici, A. Pauss, N. Mameri, Treatment of Oued El Harrach river water by electrocoagulation noting the effect of the electric field on microorganisms, J. Taiwan Inst. Chem. Eng. (2014). https://doi.org/10.1016/j.jtice.2013.10.006.

[105] E.-H. Lee, B. Chua, A. Son, Detection of Cyanobacteria in Eutrophic Water Using a Portable Electrocoagulator and NanoGene Assay, (2018). https://doi.org/10.1021/acs.est.7b05055.

[106] E.-H. Lee, B. Chua, A. Son, Detection of Cyanobacteria in Eutrophic Water

Using a Portable Electrocoagulator and NanoGene Assay, Environ. Sci. Technol. 52 (2018) 1375–1385. https://doi.org/10.1021/acs.est.7b05055.

[107] M.S. Wiederoder, S. Smith, P. Madzivhandila, D. Mager, K. Moodley, D.L. DeVoe, K.J. Land, Novel functionalities of hybrid paper-polymer centrifugal devices for assay performance enhancement, Biomicrofluidics. 11 (2017). https://doi.org/10.1063/1.5002644.

[108] R. Gorkin, J. Park, J. Siegrist, M. Amasia, B.S. Lee, J.M. Park, J. Kim, H. Kim, M. Madou, Y.K. Cho, Centrifugal microfluidics for biomedical applications, Lab Chip. 10 (2010) 1758–1773. https://doi.org/10.1039/b924109d.

[109] X. Wu, X. Huang, Y. Zhu, J. Li, M.R. Hoffmann, Synthesis and application of superabsorbent polymer microspheres for rapid concentration and quantification of microbial pathogens in ambient water, Sep. Purif. Technol. 239 (2020) 116540. https://doi.org/10.1016/j.seppur.2020.116540.

[110] S. Mukherjee, Isolation and Purification of Industrial Enzymes: Advances in Enzyme Technology, in: Adv. Enzym. Technol., Elsevier, 2019: pp. 41–70. https://doi.org/10.1016/b978-0-444-64114-4.00002-9.

[111] Y. Su, Chapter 1: Current State-of-the-art Membrane Based Filtration and Separation Technologies, in: RSC Nanosci. Nanotechnol., Royal Society of Chemistry, 2019: pp. 1–13. https://doi.org/10.1039/9781788013017-00001.

[112] Y. Zhang, C. qing Xu, T. Guo, L. Hong, An automated bacterial concentration and recovery system for pre-enrichment required in rapid Escherichia coli detection, Sci. Rep. 8 (2018). https://doi.org/10.1038/s41598-018-35970-8.

[113] M. Martín, P. Salazar, C. Jiménez, M. Lecuona, M.J. Ramos, J. Ode, J. Alcoba, R. Roche, R. Villalonga, S. Campuzano, J.M. Pingarrón, J.L. González-Mora, Rapid Legionella pneumophila determination based on a disposable core-shell Fe_3O_4@poly(dopamine) magnetic nanoparticles immunoplatform, Anal. Chim. Acta. 887 (2015) 51–58. https://doi.org/10.1016/j.aca.2015.05.048.

2. ON-FILTER PROTOCOL FOR CONCENTRATION AND DETECTION OF *Escherichia coli*

Published in Analytical and Bioanalytical Chemistry (Appendix B)

J.J. Ezenarro, N. Uria, Ó. Castillo-Fernández, N. Párraga, M. Sabrià, F.X. Muñoz Pascual, *Development of an integrated method of concentration and immunodetection of bacteria*, Anal. Bioanal. Chem. 410 (2018) 105–113.

ABSTRACT

The microbial quality of water is a key aspect to avoid environmental and public health problems. The low pathogen concentration needed to produce a disease outbreak makes essential to process large water volumes and use sensitive and specific methods such as immunoassays for their detection. In the present work, the development of a device based in microfiltration membranes to integrate the concentration and the immunodetection of waterborne bacteria is described. A microfiltration membrane treatment protocol was designed to reduce the non-specific binding of antibodies, for which different blocking agents were tested. Thus, the proof of concept of the microbial detection system was also carried out using *Escherichia coli* (*E.coli*) as bacterial pathogen model. *E.coli* suspensions were filtered through the membranes at 0.5 $mL \cdot s^{-1}$ and the *E.coli* concentration measurements were made by measuring the absorbance, at 620 nm, of the resultant product of the enzymatic reaction among the horseradish peroxidase (HRP) bonded to the antibody, and the substrate 3,3',5,5'-Tetramethylbenzidine (TMB). The results showed that the home-made concentration system together with the developed membrane treatment protocol is able to detect *E.coli* cells with a limit of detection (LoD) of about 100 CFU in 100 mL.

2.1 INTRODUCTION

The detection of pathogens present in water is an increasing concern since is one of the leading causes of mortality worldwide. So, over the recent years, there have been numerous epidemic outbreaks associated with waterborne pathogens being the cause of about 5.7% of global diseases and 4% of deaths. In this context, the determination of the microbial quality of the water has great importance to avoid environmental and public health problems [1–3].

One of the biggest hindrances of waterborne pathogens is their low occurrence. As a consequence, large sample volumes need to be processed in order to increase the sensitivity of any methodology [4–8]. Pre-concentration is the most recurrent option to overcome this problem. There are several methods such as filtration, centrifugation and immunomagnetic separation, which are used to reduce the sample volume and, therefore, increase the pathogen concentration enhancing sensitivity [9]. Additionally, the methods and techniques for the detection of pathogens in water have not significantly improved in the last years. Conventional methods as colony counting, Enzyme-Linked ImmunoSorbent Assay (ELISA) or Polymerase Chain Reaction (PCR) continue being the most commonly used [10–12]. Nevertheless, these methods have several limitations, such as large assay times, high elevated costs, or the need for qualified personnel and specialized equipment [4,11,12].

Actually, there still remains the necessity of a rapid, sensitive and inexpensive pathogen detection system. Besides to fulfill these characteristics, the developed system should be, as well, user-friendly and portable to eliminate the requirement of transporting the samples to the laboratory [13]. A promising way to address these objectives could be using the microfiltration membranes to carry out the concentration processes as well as to support the immunologic reaction. Thereby, this could engender diverse benefits such as the drastic reduction of the sample manipulation and a faster detection process since the whole process would be done on a unique device.

On the one hand, microfiltration (MF) is an easy, direct and simple form to reduce large sample volumes and concentrate the targets of interest. MF is a size-based separation process in which a porous membrane acts as barrier retaining particles

in the size range of 0.1-10 µm [14–16]. These membranes are manufactured in diverse materials and have been widely used for virus and bacteria retention [4]. On the other hand, immunoassays are widely used bioanalytical methods, in which a biological target is detected due to its specific binding with a labeled antibody [6,17]. However, the use of MF membranes as support of the immunoassays also has several drawbacks. The most important drawback to overcome is the non-specific binding of the antibodies to the membranes, which can produce false positives.

A flow-injection immunofiltration assay for detection of *E.coli* was developed by Abdel-Hamid *et al* (1999) [17]. The immunosensor consisted of an antibody-modified nylon membrane filter and a sandwich immunoassay. So, 1 mL samples were filtered at 0.12 mL·min^{-1} obtaining a detection limit of 100 cells·mL^{-1} in 30 min. Nevertheless, the water regulation standard of 0 coliforms per 100 mL set by the U.S. Environmental Protection Agency (EPA) makes still having to improve the detection limit [18,19]. Thus, the solution lies in improving the concentration step, obtaining a system able to deal with larger sample volumes and detecting coliform bacteria in a reasonable period of time.

In this paper, a device consisting on a home-made concentration system that, coupled to a direct immunoassay, is capable of retaining and detecting the bacterial cells present in water samples is developed. The device is able to deal with larger sample volumes than actually available biosensors. Additionally, the developed protocol maximally reduces the non-specific binding of antibodies to MF membranes, improving the signal-to-noise ratio being able to detect *E.coli* with a detection limit of about 1 CFU·mL^{-1}.

2.2 MATERIALS AND METHODS

Membrane materials

The analysis of the best platform to support immunologic reaction and concentration was carried out using three different membranes with a nominal pore diameter of 0.2 µm. The materials compared were nitrocellulose (NC) (Whatman Nitrocellulose, GE Health care Life science), polyvinylidene difluoride (PVDF) (Immuno-Blot PVDF

Membrane for Protein Blotting, BioRad) and polycarbonate (PC) (Whatman Nuclepore Track-Etched Polycarbonate, GE Healthcare Life science).

Blocking and washing agents

To reduce the unspecific binding of the antibody to the membranes, different blocking agents were analyzed. As blocking agents, proteins and surfactants were compared. As protein blocking agents, Bovine Serum Albumin (BSA) (Sigma-Aldrich) and Western Blocking Reagent (WBR) (Roche Life Science) at concentrations of 0.5, 1 and 2.5% of the solution volume were analyzed. Tween-20 (Tw-20) (Sigma-Aldrich) and Triton 100 (TX-100) (Sigma-Aldrich) were checked as surfactants at concentrations of 0.1, 0.25 and 1%. All reagent concentrations were selected from molecular biology protocols where these blocking agents are commonly used [20,21]. All blocking solutions were prepared using phosphate buffer saline 0.01 M (PBS) (Sigma-Aldrich) as solution-base.

Membrane blocking procedure

The different membranes (NC, PVDF, PC) were cut into 5 mm diameter discs and treated with the blocking solutions (Figure 2.1). For the blocking step, the membrane discs were put in 1.5 mL tubes containing 500 µL of each blocking reagent (Tw-20, TX-100, WBR, BSA) at different concentrations (0.1, 0.25 and 1% for detergents and 0.5, 1 and 2.5% for proteins). Tubes containing the membrane discs submerged in the corresponding solution were incubated for 2 h at 10 rpm using a rotator. In the case of PVDF membranes, due to their high hydrophobicity, a pretreatment step was needed to reduce their hydrophobicity. For this reason, before the blocking step, PVDF discs were soaked in methanol for 5 min and washed in sterilized deionized water for 5 min.

Antibodies and the immunological reaction procedure

Escherichia coli binding rabbit horseradish peroxidase-labeled polyclonal antibody (anti-*E.coli* antibody-HRP; E3500-06F, USBiologicals, Swampscott, MA, USA) was used to compare the unspecific binding to the different membrane materials (Figure 2.1).

Blocked membrane discs (see section "Membrane blocking procedure") were transferred to tubes containing 500 µL of anti-*E.coli* antibody-HRP at a final concentration of 2 $\mu g \cdot mL^{-1}$ in PBS. Membranes were incubated with the antibody for a period of 30 min in rotation at 10 rpm). After this, membranes were washed to remove the excess of antibody. For this purpose, different concentrations of Tw-20 were tested (0.05, 0.1, and 0.5% in PBS). Membranes were washed three times submerging them in tubes containing 500 µL of different washing concentrations for 5 min in rotation at 10 rpm. Finally, a last washing step was performed transferring each membrane to a tube containing 500 µL of PBS (0.01M) and incubating at 10 rpm for 5 min to remove the detergent of the membranes.

Immunologic reaction measurements

After incubations and washing processes, the amount of antibody bound to the membranes was quantified by absorbance. The 5 mm diameter membranes were placed on the bottom of the well of an ELISA 96 Microwell dish (Nunc-Immuno MicroWell 96 well, Sigma-Aldrich) and 100 µL of the colorimetric substrate Enhanced K-Blue (Neogen) were added to each well. This substrate contains 3,3',5,5'-Tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2). The reaction between HRP conjugated with the antibody and the TMB substrate makes the solution color turn into blue being the intensity of the color proportional to the amount of antibody adsorbed to the membranes. So, this was quantified by measuring absorbance at 620 nm using an ELISA reader (Multiskan EX, Thermo scientific). At minute 16, time enough to observe a stabilization of the antibody-substrate reaction, absorbance values were taken and normalized by the corresponding blank, consisting in a membrane incubated without antibodies (Figure 2.1), to compare the different blocking treatments. This resultant value was named normalized absorbance (AbsN).

$$AbsN = Abs\ (membrane + Ab) - Abs\ (membrane) \qquad \text{(Equation. 2.1)}$$

Likewise, the maximum non-specific binding (Figure. 2.1), the binding among the antibody and membranes without any type of blocking treatment, was also evaluated. By comparing the differences between treated and non-treated membranes, non-specific binding reduction could be quantified and the efficiency of

the four blocking solutions tested. All the performed measurements were carried out in triplicate and averages and standard deviations were calculated.

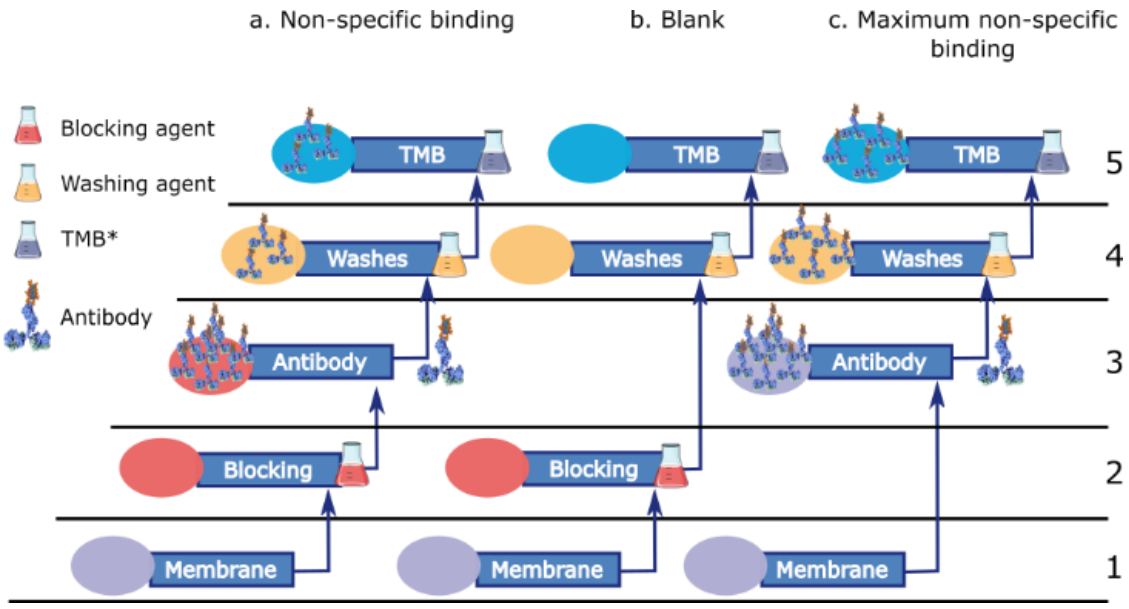

Figure 2.1 Scheme of the immunoassay protocol developed on the microfiltration membranes. The numbers indicate each step of the immunoassay: non-treated membrane (1), blocking process (2), antibody incubation (3), washing (4), and TMB reaction. The columns indicate the different procedures carried out to the analysis of antibody- membrane reaction: a non-specific binding: treatment that includes the whole process, (blocking and anti-*E. coli* (HRP-Ab) incubation), b blank: blocking treatment without anti-*E. coli* antibody to obtain the intrinsic absorbance from each membrane material, and c maximum non-specific binding obtained incubating unblocked membranes with anti-*E. coli* antibody. (TMB*; 3,3′,5,5′-tetramethylbenzidine).

Custom made concentration platform

The design of the concentration platform prototype employed for this work was carried out to integrate a sensing platform to the filtering procedure. The prototype, represented in Figure 2.2, consists of a 42 mm diameter cylinder, fabricated in polymethyl methacrylate (PMMA) divided in two parts. The upper part is formed by a reaction chamber of 20 mm of diameter and a groove of 3 mm of width to place the O-ring to ensure the water-tightness. The underside part has a planar surface provided with evacuation channels to facilitate water exit and reduce the pressure inside the holder. Additionally, both parts had threaded holes, to lock the holder and to adjust the standard Luer connectors (Plastic Value, threaded style Luer, Polypropylene)

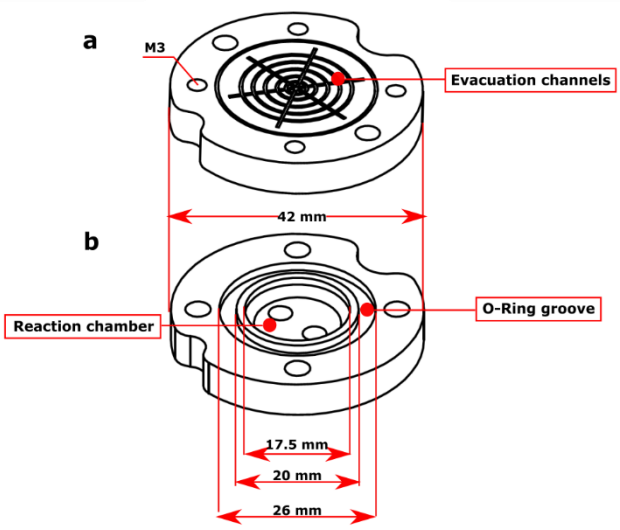

Figure 2.2 Custom-made concentration platform. a) Underside part, consisted of evacuation channels and metric 3 (M3) threaded wholes for the screws. b) Upper part, provided with a reaction chamber for the different steps involved in the immunoassay and an O-ring groove to ensure watertightness. Threaded wholes are metric 3 (M3).

Bacterial cultures

Escherichia coli (*E.coli*) ATCC 10536 strain was grown at 37 °C overnight in 5 mL of Luria-Bertani medium (LB, Sigma-Aldrich). From this overnight culture, serial dilutions of *E.coli* were prepared in tubes containing 1 mL of Ringer solution (Sodium Chloride, Sigma-Aldrich) to obtain concentrations between 10^2 and 10^6 CFU·mL^{-1}. Finally, 100 mL of water were inoculated with the desired bacterial concentration. The concentration of the overnight culture and the prepared serial dilutions were estimated by plate count in LB agar. The colony forming units per milliliter (CFU·mL^{-1}) were calculated by the following formula:

$$\text{CFU·mL}^{-1} = \frac{\text{number of colonies on plate}}{(\text{dilution factor x seeding volume})} \quad \text{(Equation 2.2)}$$

Filtration processes

All the filtration processes were carried out by placing 25 mm diameter membranes on the concentration platform and passing the sample through at a flow rate of 0.5 mL·s^{-1}, employing a peristaltic pump (Gilson Miniplus3).

Testing of different blocking phases

In order to find out the best blocking procedure to enhance bacterial detection, the efficiency of different blocking steps was analyzed. With this aim, water samples with a final *E.coli* concentration of 10^6 CFU in a volume of 100 mL were filtered through 25 mm NC and PC membranes blocked with Tw-20 at 1% or WBR at 2.5%.

Regarding the blocking phases, 2 different procedures were used. In Procedure 1, membranes were blocked for 2 h and after this, bacterial samples were filtered. In Procedure 2 membranes were blocked for 2 h before and after sample filtration. In all cases, the membranes were blocked following procedure described in section "Membrane blocking procedure". A control was also carried out in which 100 mL of sterile distilled water were filtered.

Membranes treated with each different blocking step were cut into four 5 mm diameter discs as replicates. Three discs were transferred to tubes containing 500 µL of anti-*E.coli* antibody-HRP, and the fourth replicate was used to analyze the signal of the proper membranes (blank).

***E.coli* detection and quantification calibration curve**

In order to verify the capacity of the developed system to detect and quantify *E.coli*, samples with a final concentration between 10^2 and 10^6 total cells in 100 mL of sterile distilled water were prepared. *E.coli* solutions were filtered through membranes blocked before and after filtration. Three whole membranes (25 mm) of each bacterial concentration as replicates were transferred to plates containing 2 mL of anti-*E.coli* antibody-HRP at a final concentration of 5 $\mu g \cdot mL^{-1}$ in PBS. Membranes and the antibody were incubated for a period of 30 min in agitation at 30 rpm. After this, membranes were washed three times to remove the excess of antibody. For this, membranes were transferred to plates containing 2 mL Tw-20 at 0.5 % for 5 min in rotation at 30 rpm. A last washing step was performed transferring each membrane to 2 mL of PBS (0.01M) and incubating at 30 rpm for 5 min to remove the detergent of the membranes. Finally, membranes were placed on a new plate and 500 µL of the colorimetric substrate Enhanced K-Blue were added to each well. After 16 min of reaction in agitation at 80 rpm, 100 µL of the resultant solution were transferred to an ELISA 96 Microwell plate. Thus, absorbance at 620 nm was measured by using

an ELISA reader. Finally, the detection limit was determined by the following equation:

$$\text{Limit of Detection (LoD)} = \text{Blank signal} + 3*SD \qquad \text{(Equation. 2.3)}$$

2.3 RESULTS AND DISCUSSION

Effect of the blocking and washing solutions on the reduction of non-specific binding

It is well known that an important aspect that determines the specificity and sensitivity of a detection method is the signal-to-noise ratio. That is to say, the measure used to compare the level of a desired signal to the level of background noise or an unwanted signal [22]. For this reason, as well as it occurs with immunoassays, membranes need to be blocked to avoid unspecific antibody binding [23], being the choice of the blocking agent critical in order to decrease false positives. For this reason, one of the principal objectives of this work was to define an optimized protocol for the reduction of non-specific binding of antibody to membranes. With this aim, the efficiency of different blocking agents to reduce non-specific membrane/antibody binding was compared in our filtration system.

Protein and detergent-based solutions are the most employed blocking agents in assays that imply the use of membranes as support of an immunoreaction processes such as immunoblots, ELISA or Enzyme-linked ImmunoFiltration Assay (ELIFA) [20,24–29]. However, since a perfect and standard blocking agent does not exist, the selection of the best solution needs to be optimized to each method [20,30]. In this way, 2 protein (BSA and WBR) and 2 detergent (Tw-20 and TX-100) based solutions were analyzed as blocking reagents at 3 different concentrations selected from several immunoassay protocols available in literature (0.5, 1, 2.5 % for proteins [20,28] and 0.1, 0.25, and 1% for detergents [31–33]).

In addition to the blocking processes, several steps of membrane washing are also important to remove the excess of antibody, which remain deposited on the surface despite to the blocking treatment. Normally, washing solutions are prepared with low

detergent concentrations such as Tween-20 (Tw-20) to rinse the antibody deposited on the membrane by breaking weak bonds among both [28,34]. So, 3 concentrations of Tw-20 (0.05, 0.25, and 0.5%) were tested as washing solutions after incubating the membrane with the antibody. Tw-20 was chosen for this aim as it has been commonly used for these purposes in immunoassays [35–38]. The results were compared to the signal obtained with non-treated membranes, which shows the maximum non-specific binding.

The absorbance data obtained by the 3 membrane materials have been illustrated in Figure 2.3. In all cases, the normalized absorbance (AbsN) (Z-axis) in relation to the washing (X-axis) and blocking (Y-axis) solution concentration is represented. All data obtained in these experiments are available in Appendix A (Table 1).

Protein based blocking solutions

In reference to BSA (Figure 2.3a), a reduction of the non-specific binding was appreciated after membrane treatment for all the materials. In general the results indicated that the minimum BSA concentration used was enough to block the membranes and, despite using higher concentrated solutions, differences were not observed.

In detail, PC (Figure 2.3a1; Appendix A, Table 1a) showed the best results with a non-specific binding reduction close to 100%. The most interesting point is that this reduction was obtained for all concentrations and washing steps. In contrast, the results obtained by NC and PVDF membranes showed that non-specific binding reduction was related to the washing solution concentration (Figure. 2.3a2, Figure 2.3a3; Appendix A, Table 1a). So, while non-treated membranes of NC and PVDF had an AbsN average value of 1.27 and 1.81, after washing with Tw-20 at 0.5% values were reduced to 0.24 and 0.14 respectively (Figure 2.3). Therefore, in general the best results were obtained by the washing solution concentration of 0.5% Tw-20 reducing the unspecific binding about 78% and 89%, in the case of NC and PVDF respectively for all BSA concentrations (Figure 2.3a2, Figure 2.3a3; Appendix A, Table 1a). The differences observed between NC, PVDF and PC could be related to their structures. NC (Appendix A, Figure 1) and PVDF (Appendix A, Figure 2) membranes are a complex mesh while so, the blocking solution could not be able to cover all the fibers of their structures. On the other hand, PC (Appendix A, Figure 3) is a perforated flat

polymer surface, which enables the formation of a uniform layer of blocking agent with better results.

In the case of WBR, which is represented in Figure 2.3b, a great efficiency of this blocking agent was observed for the three membrane materials presenting the lowest non-specific binding (Figure 2.3b; Appendix A, Table 1b). Thus, the lowest concentration of WBR was enough to reduce the non-specific binding of the antibody. As opposed to the case of BSA, washing steps were not necessary to improve this reduction since absorbance values close to zero were measured for the tested WBR and Tw-20 washing concentrations.

Detergent based blocking solutions

In Figure 2.3c the results obtained for different membrane materials treated with Tw-20 are shown. In all cases, the reduction of the non-specific binding is related to the Tw-20 concentration. The best performance was obtained by the 1% concentration with a final reduction of the non-specific binding close to 100% for PC membranes (from around 1.7 absorbance value of maximum non-specific binding to 0.04) (Figure 2.3c1) and 90% for NC and PVDF membranes (Figure 2.3c2, Figure 2.3c3) (from around 1.8 to 0.2 in the case of PVDF and from 1.3 to 0.25 in the case of NC). In relation with the washing buffer concentration used, it was observed that higher concentrations did not reduce the non-specific values regardless of the blocking agent and concentration used. (Figure 2.3c; Appendix A, Table 1c).

In contrast, TX-100 was the most inefficient blocking reagent for the three materials. In fact, TX-100 had no effect on membrane/antibody binding reduction and all the measured values were equal (Appendix A, Table 1d). Probably, TX-100 was not able to interact with the functional groups of the membranes and as a consequence the unspecific binding of antibodies was not reduced.

In general, the results showed that the highest concentration of all blocking agents leaded to the best results of unspecific binding reduction for all membrane materials. This indicates that the same blocking concentrations employed in immunoblot assays are necessary in this system since high concentrations of the blocking solutions are essential to avoid the unspecific binding membrane/antibody [20,28,32].

Washing steps

The effect of the washing steps was also analyzed. The best Tw-20 concentration to perform the washing steps was 0.5 %. As mentioned before, these could be appreciated quite well for the case of BSA. Some other authors also described that, despite lower concentrations are usually employed in immunoblotting protocols, a major decrease of this type of binding is obtained when 0.5 % Tw-20 is used as washing solution [29,39]. For these reason, this Tw-20 concentration was chosen as washing solution for the following experiments.

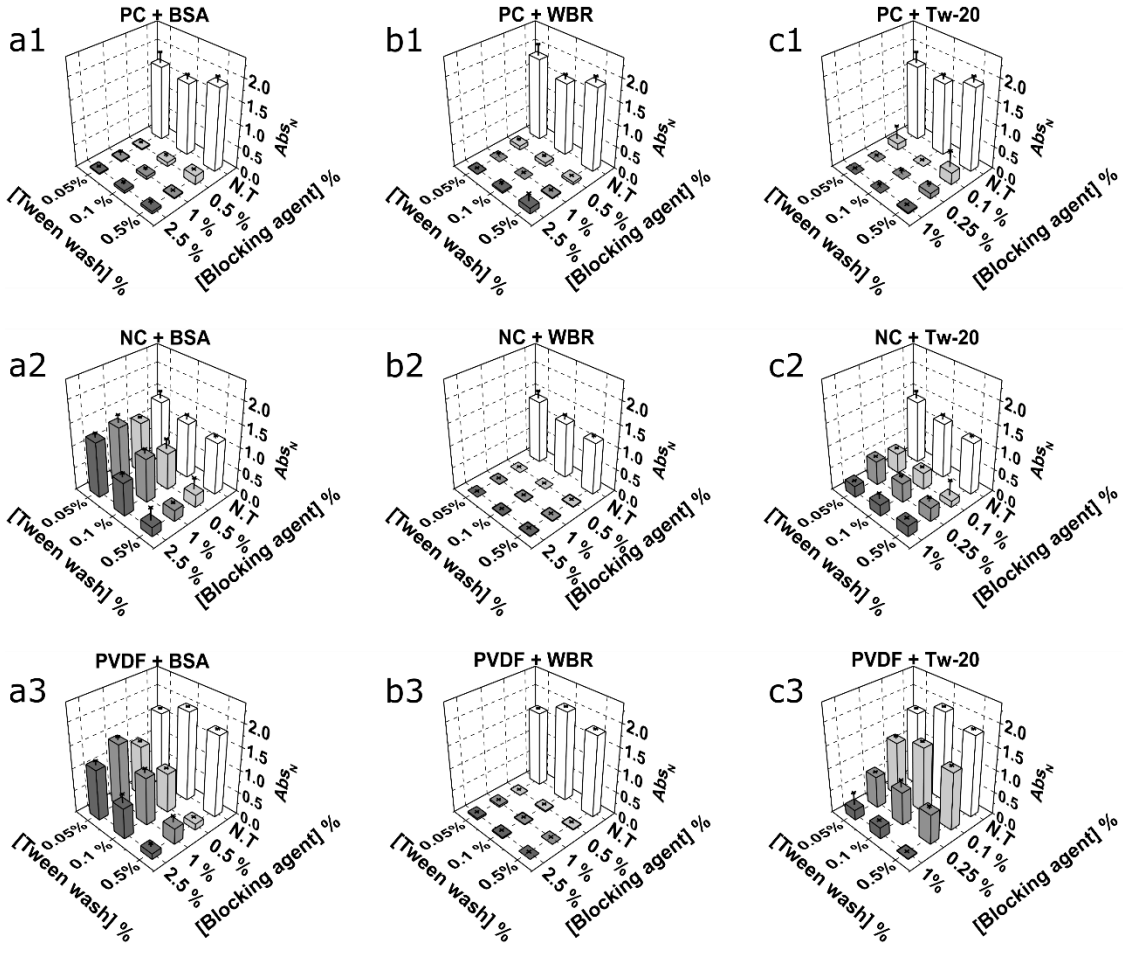

Figure 2.3 Representation of the normalized absorbance (AbsN) measured for the different blocking treatments BSA (a), WBR (b), and Tw-20 (c) and the three membrane materials PC (1), NC (2), and PVDF (3). In all cases, the normalized absorbance (AbsN) is represented (Z-axis) in relation to the washing concentration (X-axis) and the blocking agent (Y-axis) solution concentration.

Comparison of blocking phases

In this study, the results obtained with clean and bacterial samples after different steps of blocking (before and before-after the filtration process) were tested. For this, *E.coli* was used as model of fecal bacteria since it has been used as fecal contamination indicator since the 19th century [2,11,40–42]. Additionally, in the light of the previous results, WBR at 2.5% and Tw-20 at 1% were selected as blocking agents and NC and PC as membrane materials. PVDF membranes were discarded for these experiments since their hydrophobic nature caused problems during water samples filtration.

First of all, a blocking step previous to the sample filtration was analyzed (Figure 2.4a, 4c). On the one hand, NC membranes treated with WBR 2.5% showed low absorbance values when samples without *E.coli* were filtered (Abs=0.075) with respect to non-treated membranes (Abs=0.669). This indicated that WBR reminded covering the membranes after sample filtration and therefore, a low non-specific binding of the antibody was observed. In contrast, when Tw-20 was used as the blocking agent, the absorbance value obtained by the NC membranes treated (Abs=0.745) and no-treated (Abs=0.669) was very similar. So, non-specific binding of the antibody to the membrane was not reduced, suggesting that blocking was lost during the filtration performance. This could be due to the fact that Tween-20 is a detergent, and the passage of water could remove it from the membranes.

When samples containing *E.coli* were filtered, no increase in absorbance values was observed (Abs_{WBR}=0.075, Abs_{Tw}= 0.592) independently of the type of blocking agent used. These could be attributed to the morphology of the membranes being of the great importance for the detection system. NC membranes, have a fluffy structure, formed by a complex mesh without straight pores with cavities and large surface areas [32,43] (Appendix A, Figure 1). For this reason, the pressure exerted by the water flow (30 mL· min^{-1}) seemed to make the cells penetrate into their structure and not remaining on the surface, and thus, hinder the detection.

On the other hand, the absorbance values measured for PC membranes treated with WBR and Tw-20 were higher than 1.4 and 1.6 respectively, even when the filtered samples were not inoculated with *E.coli*. PC membranes have flat surfaces with straight-through pores being ideal substrates for rapid microbiological test methods (Appendix A, Figure 3), such as methods employing optical sensors [44,45]. However, the fact that water samples need to be filtered through them to retain the bacteria,

caused the elimination of the blocking treatment and as a result the non-specific binding of the antibody to the membrane increased (Figure 2.4c). In general, the results showed that the morphology of the membranes may have an important role and suggested that the water flow through the membranes affected the blocking performance.

Since the blocking step previous to the filtration resulted to be insufficient, a second post-filtration blocking step was tested (Figure 2.4b). The results obtained for the membranes treated with the blocking agents before and after the sample filtration are shown in Figure 2.4c.

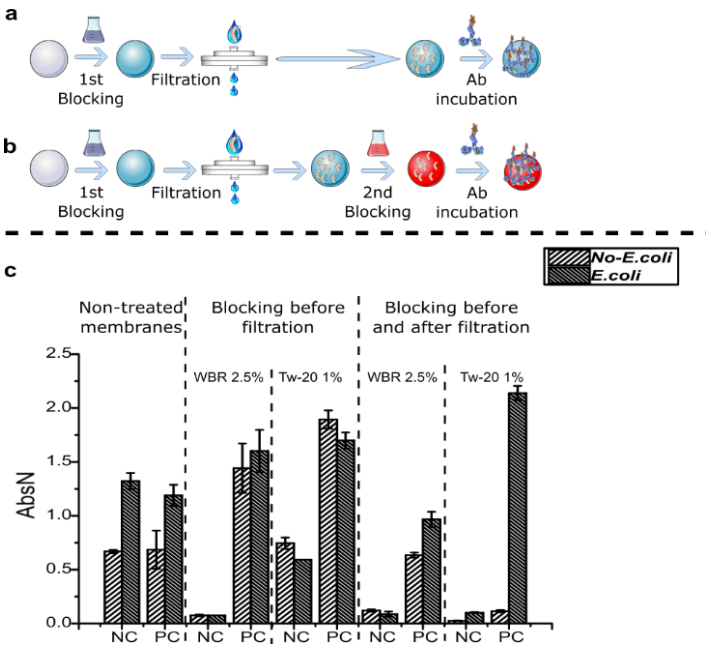

Figure 2.4 Blocking phase comparison. a) Scheme of the membrane blocking treatment before the filtration process. b) Scheme of the membrane blocking treatment before and after the filtration process. c) Normalized absorbance values obtained by membranes treated with WBR 2.5% and Tw-20 1% before, and before and after filtration. Both cases are compared to the results obtained for the non-treated membranes

In terms of the best material, the results showed that NC membranes blocked twice with WBR 2.5% or Tw-20 1% were not able to distinguish among clean water sample (No-*E.coli*) (Abs_{WBR}=0.121 and Abs_{Tw}=0.024) and a bacterial suspension (*E.coli*) (Abs_{WBR}=0.088 and Abs_{Tw}=0.1). This could be related again to the structure of NC, which allows bacterial cells to penetrate inside their structure helped by the pressure exerted by the water flow and thus, difficult the antibody binding to the cells and

hinder the detection. In contrast, for PC membranes, an absorbance value of 0.669 was obtained with the blank (No-*E.coli*) when the membranes were not subjected to a blocking treatment (No-treated). This value was reduced only by a 5% when WBR was used as blocking agent, while the value for membranes treated with Tw-20 was reduced by about 83%. Besides, when the filtered sample was an *E.coli* suspension, the signal obtained with membranes treated with Tw-20 was much higher (2.139) than the signal obtained with membranes treated with WBR (0.967). These results suggested that the WBR blocking step performed after *E.coli* suspension filtration, affects negatively to the detection. It seems that WBR hinders antibody/*E.coli* binding. As a result, the signal difference obtained between the blank (No-*E.coli*) and the *E.coli* suspension for WBR was about 34%, while in case of Tw-20 no affections were observed and an absorbance value difference of 94% was obtained. So, PC membranes blocked with Tw-20 showed a wider range of sensitivity to allow the distinction of different and lower bacterial concentrations and therefore, they were selected as the best material and treatment.

E.coli detection and quantification calibration curve

Finally, a last experiment was carried out with PC membranes treated with the double step blocking protocol, using Tw-20 at 1% as blocking agent, to test the capacity of the system to detect and quantify different concentrations of *E.coli*.

In Figure 2.5, the relation between the absorbance and the different *E.coli* concentrations analyzed is shown. It could be appreciated that as higher was the *E.coli* concentration present in the sample, higher was the obtained absorbance value at 620 nm. Moreover, this calibration curve maintained a good linearity with an R^2 value of 0.99. Abdel-Hamid *et al* (1999) developed an immunosensor based in nylon membrane filters able to obtain results in 30 min after filtering 1 mL at 0.12 mL·min^{-1} [17], and Eltzovand Marks [46] developed a flow stacked immunoassay consisting of different nitrocellulose pads with various components. Both methods obtained a threshold sensitivity of 10^2 CFU·mL^{-1}. These methods succeeded in overcoming large assay times, but continued working with low sample volumes. Dharmasiri *et al* (2010), developed a microfluidic chip followed by quantitative PCR to cell enrichment and detection with a LoD of 6-10 CFU from 100 mL samples in 5 h. However, the 100 mL need to be filtered to reduce de volume to 1 mL before using the microfluidic chip [47]. In contrast, our system takes only few ours (2-3 h) overcoming large assay

times, is able to handle with larger sample volumes (100 mL) working at high flow rates (30 mL·min^{-1}) without any pre-processing step. Additionally, the system presents a LoD of about 10^2 *E.coli* cells in 100 mL with a variability of 14%. Besides, the integration of sample concentration and immunoassay processes into a single device could endanger benefits as a lower reactive consume and an easier automation.

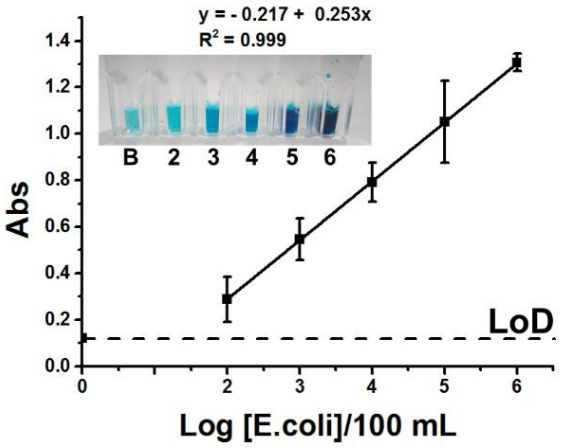

Figure 2.5 Relationship between the absorbance measured at 620 nm (min 16 of reaction) and the *E. coli* concentration present in 100 mL water samples. The dashed line corresponds to the limit of detection (LoD) of the system.

2.4 CONCLUSIONS

In this work a new waterborne pathogen concentration and detection system is presented. With the aim of the integration of concentration and immunodetection processes into a single device, a home-made filter holder to support microfiltration membrane was designed. Additionally, a protocol to reduce the non-specific binding of antibody to microfiltration membranes allowing bacterial detection was developed. This way filtration through polycarbonate membranes blocked with Tween 20 at 1 % before and after filtration processes, and washed with Tw-20 at 0.5% was the most effective membrane treatment permitting the *E.coli* detection and quantification, detecting about 10^2 *E.coli* cells in 100 mL of water (1 cell·mL^{-1}). The obtained

calibration curve clearly showed the detection capability of this protocol and confirmed that the developed detection system could be used for the determination of bacterial concentrations in water samples. Additionally, future work is focused on fully integrating the protocol into the holder and automating the system in order to achieve a fast and simple bacteria detection device.

REFERENCES

[1] Y. Delaedt, A. Daneels, P. Declerck, J. Behets, J. Ryckeboer, E. Peters, F. Ollevier, The impact of electrochemical disinfection on Escherichia coli and Legionella pneumophila in tap water, Microbiol. Res. 163 (2008) 192–199. https://doi.org/10.1016/j.micres.2006.05.002.

[2] A. Golberg, G. Linshiz, I. Kravets, N. Stawski, N.J. Hillson, M.L. Yarmush, R.S. Marks, T. Konry, Cloud-enabled microscopy and droplet microfluidic platform for specific detection of Escherichia coli in water, PLoS One. 9 (2014) e86341. https://doi.org/10.1371/journal.pone.0086341.

[3] F. Tian, J. Lyu, J. Shi, F. Tan, M. Yang, A polymeric microfluidic device integrated with nanoporous alumina membranes for simultaneous detection of multiple foodborne pathogens, Sensors Actuators, B Chem. 225 (2016) 312–318. https://doi.org/10.1016/j.snb.2015.11.059.

[4] J. Shah, S. Chemburu, E. Wilkins, I. Abdel-Hamid, Rapid Amperometric Immunoassay for Escherichia coli Based on Graphite Coated Nylon Membranes, Electroanalysis. 15 (2003) 1809–1814. https://doi.org/10.1002/elan.200302791.

[5] P. Leonard, S. Hearty, J. Brennan, L. Dunne, J. Quinn, T. Chakraborty, R. O'Kennedy, Advances in biosensors for detection of pathogens in food and water, Enzyme Microb. Technol. 32 (2003) 3–13. https://doi.org/10.1016/S0141-0229(02)00232-6.

[6] I.A. Darwish, Immunoassay Methods and their Applications in Pharmaceutical Analysis: Basic Methodology and Recent Advances., Int. J. Biomed. Sci. 2 (2006) 217–35.

[7] X. Li, E. Ximenes, M.A.R. Amalaradjou, H.B. Vibbert, K. Foster, J. Jones, X. Liu, A.K. Bhunia, M.R. Ladisch, Rapid Sample Processing for Detection of Food-Borne Pathogens via Cross-Flow Microfiltration, Appl. Environ. Microbiol. 79 (2013) 7048–7054. https://doi.org/10.1128/AEM.02587-13.

[8] U. Dharmasiri, M.A. Witek, A.A. Adams, S.A. Soper, Microsystems for the capture of low-abundance cells., Annu. Rev. Anal. Chem. 3 (2010) 409–31. https://doi.org/10.1146/annurev.anchem.111808.073610.

[9] R.T. Noble, S.B. Weisberg, A review of technologies for rapid detection of bacteria in recreational waters, J. Water Health. 3 (2005) 381–392. https://doi.org/10.2166/wh.2005.051.

[10] J.-T. Kuo, C.-Y. Cheng, H.-H. Huang, C.-F. Tsao, Y.-C. Chung, A rapid method for the detection of representative coliforms in water samples: polymerase chain reaction-enzyme-linked immunosorbent assay (PCR-ELISA), J. Ind. Microbiol. {&} Biotechnol. 37 (2010) 237–244. https://doi.org/10.1007/s10295-009-0666-0.

[11] A. Rompré, P. Servais, J. Baudart, M.-R. De-Roubin, P. Laurent, Detection and enumeration of coliforms in drinking water: current methods and emerging approaches, J. Microbiol. Methods. 49 (2002) 31–54. https://doi.org/10.1016/S0167-7012(01)00351-7.

[12] O. Lazcka, F.J. Del Campo, F.X. Muñoz, Pathogen detection: A perspective of traditional methods and biosensors, Biosens. Bioelectron. 22 (2007) 1205–1217. https://doi.org/10.1016/j.bios.2006.06.036.

[13] V. Velusamy, K. Arshak, O. Korostynska, K. Oliwa, C. Adley, An overview of foodborne pathogen detection: In the perspective of biosensors, Biotechnol. Adv. 28 (2010) 232–254. https://doi.org/10.1016/j.biotechadv.2009.12.004.

[14] A. Saxena, B.P. Tripathi, M. Kumar, V.K. Shahi, Membrane-based techniques for the separation and purification of proteins: An overview, Adv. Colloid Interface Sci. 145 (2009) 1–22. https://doi.org/10.1016/j.cis.2008.07.004.

[15] J.A. Suarez, J.M. Veza, Dead-end microfiltration as advanced treatment for wastewater, Desalination. 127 (2000) 47–58. https://doi.org/10.1016/S0011-9164(99)00191-5.

[16] C. Velasco, J.I. Calvo, L. Palacio, J. Carmona, P. Prádanos, A. Hernández, Flux kinetics, limit and critical fluxes for low pressure dead-end microfiltration. The case of BSA filtration through a positively charged membrane, Chem. Eng. Sci. 129 (2015) 58–68. https://doi.org/10.1016/j.ces.2015.02.003.

[17] I. Abdel-Hamid, D. Ivnitski, P. Atanasov, E. Wilkins, Flow-through immunofiltration assay system for rapid detection of E. coli O157:H7, Biosens. Bioelectron. 14 (1999) 309–316. https://doi.org/10.1016/S0956-5663(99)00004-4.

[18] R.M. Maier, I.L. Pepper, C.P. Gerba, Environmental microbiology, Elsevier/Academic Press, 2009.

[19] R.A. Barrell, P.R. Hunter, G. Nichols, Microbiological standards for water and their relationship to health risk., Commun. Dis. Public Heal. 3 (2000) 8–13. http://www.ncbi.nlm.nih.gov/pubmed/10743312 (accessed December 20, 2016).

[20] J. Gibbs, M.E. Kennebunk, Effective blocking procedures, ELISA Tech. Bull. Kennebunk, ME Corning Inc. Life Sci. (2001).

[21] M. Johnson, Detergents: Triton X-100, Tween-20, and More, Mater. Methods. 3 (2013) 163. https://doi.org/10.13070/mm.en.3.163.

[22] Ó. Castillo-Fernandez, N. Uria, F.X. Muñoz, A. Bratov, Cell Concentration Systems for Enhanced Biosensor Sensitivity, in: Biosens. - Micro Nanoscale Appl., InTech, 2015. https://doi.org/10.5772/61088.

[23] D. Huber, J. Rudolf, P. Ansari, B. Galler, M. Führer, C. Hasenhindl, S. Baumgartner, Effectiveness of natural and synthetic blocking reagents and their application for detecting food allergens in enzyme-linked immunosorbent assays, Anal. Bioanal. Chem. 394 (2009) 539–548. https://doi.org/10.1007/s00216-009-2698-8.

[24] J.E. Butler, Solid Supports in Enzyme-Linked Immunosorbent Assay and Other Solid-Phase Immunoassays, Methods. 22 (2000) 4–23. https://doi.org/10.1006/meth.2000.1031.

[25] S. Zampieri, A. Ghirardello, A. Doria, M. Tonello, R. Bendo, K. Rossini, P.F. Gambari, The use of Tween 20 in immunoblotting assays for the detection of autoantibodies in connective tissue diseases, J. Immunol. Methods. 239 (2000) 1–11. https://doi.org/10.1016/S0022-1759(00)00168-X.

[26] J.M. Gershoni, G.E. Palade, Protein blotting: Principles and applications, Anal. Biochem. 131 (1983) 1–15. https://doi.org/10.1016/0003-2697(83)90128-8.

[27] E. Güven, K. Duus, M.C. Lydolph, C.S. Jørgensen, I. Laursen, G. Houen, Non-specific binding in solid phase immunoassays for autoantibodies correlates with inflammation markers, J. Immunol. Methods. 403 (2014) 26–36. https://doi.org/10.1016/j.jim.2013.11.014.

[28] P.T. Scientific, Western Blotting Handbook and Troubleshooting Guide, Pierce Thermo Sci. (2004) 1–52.

[29] O.E. Ijsselmuiden, P. Herbrink, M.J.M. Meddens, B. Tank, E. Stolz, R.V.W. Van Eijk, Optimizing the solid-phase immunofiltration assay. A rapid alternative to immunoassays, J. Immunol. Methods. 119 (1989) 35–43. https://doi.org/10.1016/0022-1759(89)90378-5.

[30] J.E. Butler, Immunochemistry of solid-phase immunoassay, CRC Press, 1991.

[31] B. Batteiger, W.J. Newhall V, R.B. Jones, The use of tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes, J. Immunol. Methods. 55 (1982) 297–307. https://doi.org/10.1016/0022-1759(82)90089-8.

[32] W.T. Chen, R.L. Hendrickson, C.P. Huang, D. Sherman, T. Geng, A.K. Bhunia, M.R. Ladisch, Mechanistic study of membrane concentration and recovery of Listeria monocytogenes, Biotechnol. Bioeng. 89 (2005) 263–273. https://doi.org/10.1002/bit.20256.

[33] W.L. Hoffman, A.A. Jump, Tween 20 removes antibodies and other proteins from nitrocellulose, J. Immunol. Methods. 94 (1986) 191–196. https://doi.org/10.1016/0022-1759(86)90232-2.

[34] P. Esser, M. Sc, S. Scientist, T. Fisher, Blocking Agent and Detergent in ELISA, Time. (1991) 1–4.

[35] M. Steinitz, Quantitation of the blocking effect of tween 20 and bovine serum albumin in ELISA microwells., Anal. Biochem. 282 (2000) 232–238. https://doi.org/10.1006/abio.2000.4602.

[36] A.L. De Blas, H.M. Cherwinski, Detection of antigens on nitrocellulose paper immunoblots with monoclonal antibodies, Anal. Biochem. 133 (1983) 214–219. https://doi.org/10.1016/0003-2697(83)90245-2.

[37] M.F. Clark, A.N. Adams, Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses., J. Gen. Virol. 34 (1977) 475–83. https://doi.org/10.1099/0022-1317-34-3-475.

[38] K.J. Hamblett, P.D. Senter, D.F. Chace, M.M.C. Sun, J. Lenox, C.G. Cerveny, K.M. Kissler, S.X. Bernhardt, A.K. Kopcha, R.F. Zabinski, D.L. Meyer, J.A.

Francisco, Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate., Clin. Cancer Res. 10 (2004) 7063–70. https://doi.org/10.1158/1078-0432.CCR-04-0789.

[39] M. Wu, P.G. Stockley, W.J. Martin, An improved western blotting technique effectively reduces background, Electrophoresis. 23 (2002) 2373–2376. https://doi.org/10.1002/1522-2683(200208)23:15<2373::AID-ELPS2373>3.0.CO;2-W.

[40] M.A. Croxen, R.J. Law, R. Scholz, K.M. Keeney, M. Wlodarska, B.B. Finlay, Recent Advances in Understanding Enteric Pathogenic Escherichia coli, Clin. Microbiol. Rev. 26 (2013) 822–880. https://doi.org/10.1128/CMR.00022-13.

[41] F. Hai, T. Riley, S. Shawkat, S. Magram, K. Yamamoto, Removal of Pathogens by Membrane Bioreactors: A Review of the Mechanisms, Influencing Factors and Reduction in Chemical Disinfectant Dosing, Water. 6 (2014) 3603–3630. https://doi.org/10.3390/w6123603.

[42] D. WANG, W. FIESSEL, Evaluation of media for simultaneous enumeration of total coliform and Escherichia coli in drinking water supplies by membrane filtration techniques, J. Environ. Sci. 20 (2008) 273–277. https://doi.org/10.1016/S1001-0742(08)60043-1.

[43] X. Wang, L. Zhang, Q. An, H. Chen, Morphology and formation mechanism of poly(vinylidene fluoride) membranes prepared with immerse precipitation: Effect of dissolving temperature, J. Macromol. Sci. Part B Phys. 48 (2009) 696–709. https://doi.org/10.1080/00222340902958950.

[44] M. Thompson, R. Bruce Lennox, R.A. McLelland, Structure and electrochemical properties of microfiltration filter-lipid membrane systems, Anal. Chem. 54 (1982) 76–81. https://doi.org/10.1021/ac00238a024.

[45] R. V. Levy, M.W. Jornitz, Types of filtration, Adv. Biochem. Eng. Biotechnol. 98 (2006) 1–26. https://doi.org/10.1007/b104242.

[46] E. Eltzov, R.S. Marks, Miniaturized Flow Stacked Immunoassay for Detecting Escherichia coli in a Single Step, Anal. Chem. 88 (2016) 6441–6449. https://doi.org/10.1021/acs.analchem.6b01034.

[47] U. Dharmasiri, M.A. Witek, A.A. Adams, J.K. Osiri, M.L. Hupert, T.S. Bianchi, D.L. Roelke, S.A. Soper, Enrichment and detection of escherichia coli

O157:H7 from water samples using an antibody modified microfluidic chip, Anal. Chem. 82 (2010) 2844–2849. https://doi.org/10.1021/ac100323k.

3. ON-FILTER PROTOCOL FOR CONCENTRATION AND DETECTION OF *Legionella pneumophila*

Partially published in Talanta (Appendix B)

N. Párraga-Niño, S. Quero, A. Ventós-Alfonso, N. Uria, O. Castillo-Fernandez, **J.J. Ezenarro**, F.X. Muñoz, M. Garcia-Nuñez, M. Sabrià, *New system for the detection of Legionella pneumophila in water samples*, Talanta. 189 (2018) 324–331

ABSTRACT

Waterborne pathogens are a global concern for public health worldwide. Despite continuing efforts to maintain water safety, water quality is still affected by deterioration and pollution. *Legionella pneumophila* colonizes man-made water systems and can infect humans, causing Legionnaire's disease (LD) pneumonia. The prevention of LD is a public health issue and requires specific systems to control and detect these microorganisms. Culture plate is the only technique currently approved but requires more than 10 days to obtain results. A rapid test that informs in hours about the presence of *Legionella pneumophila* in water samples will improve the control of this pathogen colonization. In order to control colonization by *L. pneumophila* we developed a membrane filter method to capture and immunodetect this microorganism in water samples. This membrane filter is used to retain the bacteria using a nitrocellulose disc inside a home-made cartridge. Subsequently, we perform the immunodetection of the bacteria retained in the nitrocellulose (blocking, antibody incubation, washings and developing). On comparing our test with the gold standard, the most important finding is the considerable reduction in time maintaining the same detection limit. This rapid test is easily automated for *L. pneumophila* detection allowing comprehensive surveillance of *L. pneumophila* in water facilities and reducing the variability in the analyses due to the low need for manipulation. Moreover, corrective measures may be applied the same day of the analysis. This method considerably reduces the detection time compared with the conventional, gold-standard detection culture method that requires more than 10 days, being decisive to prevent outbreaks.

3.1 INTRODUCTION

Waterborne pathogens and related diseases are a major public health concern worldwide. The impact of these pathogens is not only related to the associated mortality and morbidity, social impact and economic losses by prevention and treatment. The etiological agents of waterborne diseases are categorized as bacteria, viruses and parasites. Therefore, the detection of waterborne bacteria is of great importance to verify the safety in potable water.

Legionella pneumophila is the causative agent of Legionnaire's disease (LD), which has a great impact on health. LD is a disease of compulsory declaration in Spain and other countries. A total of 6573 confirmed cases of LD were declared in 2015 by the 29 member states of the European Union, and Iceland and Norway (European Centre for Disease Prevention and Control), with a case fatality rate of 10% [1].

Legionella is a Gram-negative bacterium, which is ubiquitous in freshwater habitats, including lakes, streams, ponds, rivers and soil environments [2]. Apart from natural environments, *Legionella* can colonize man-made water systems where it can grow uncontrollably and be transmitted to humans by inhalation or microaspiration [3] of aerosols generated by showers, faucets, cooling towers, whirlpool spas and fountains. *Legionella* causes LD, which is manifested as pneumonia or a mild nonpneumonic febrile illness called Pontiac fever [4]. There are over 60 species of *Legionella* [5] and *L. pneumophila* has 16 serogroups. However, *L. pneumophila* serogroup 1 (Sg 1) accounts for the majority of European and American clinical isolates [6].

It is difficult to detect and identify *Legionella* isolates from environmental water samples, and the procedure may be long and complex due to the presence of other bacteria with higher growth rates disguising *Legionella* presence. The identification of *L. pneumophila* is based on immunological techniques requiring qualified personnel and up to 10 days to obtain results.

Concentration by filtration overcomes limitations in samples with low bacterial load. The membrane retains bacteria, and these are seeded in a selective medium [7,8]. Culture-based methods are the conventional methods used to determine the presence of bacteria in water samples. There are different mediums to grow and/or selectively grow bacteria such as nutritive medium brain heart infusion (BHI) broth or selective buffered charcoal yeast extract (BCYE), which is specific for *Legionella*

growth. However, culture-based methods have several limitations including: contamination with non-*Legionella* species, laborious and time-consuming protocols and failure to detect viable but non-culturable (VBNC) cells [9]. It is important to detect VBNC since although they are not culturable, their metabolism can be reactivated leading to recolonization of systems and a switch to an infective state.

The main characteristics needed for an effective *L. pneumophila* detection method in water samples are: rapid results, low cost, no need for qualified personnel (fully automated or portable system) and a detection limit similar to the standard method (culture plate). A method providing all these features would allow better water surveillance and more frequent use would avoid the appearance of outbreaks since an alarm would be generated with the presence of *Legionella.*

Several methods have been developed for the rapid detection of *L. pneumophila* [10–13]. These approaches can be classified into nucleic-based, immunology-based methods, such as isothermal amplification, quantitative polymerase chain reaction (qPCR), immunomagnetic separation, and antibody detection. These methods are specific and time-effective for the diagnosis of waterborne bacterial diseases, although they do present some limitations [14]. Nucleic-based methods are expensive and sensitive to PCR inhibitors, purified DNA is needed and expensive equipment is required to perform the analyses. Immunological-based methods present high rates of cross-reactivity, false-negative results and have low sensitivity.

The objective of this study was to develop an integrated on-filter concentration and immunodetection protocol for the detection of *L. pneumophila*. The use of microfiltration membranes offers the possibility of processing large sample volumes allowing to have an increased sensitivity reaching low detection limits. Moreover the monoclonal antibody selected from a previous study [15], in contrast to other detection systems that are available in the market, only able to detect *L. pneumophila* Sg 1 [16,17], provides good specificity and to all *L. pneumophila* serogroups with sensitivity, with low contaminant bacteria recognition. Moreover, it has been demonstrated that the whole assay can be carried out within a home-made device, which could lead to a totally automated detection system.

3.2 MATERIAL AND METHODS

3.2.1. Reagents

Three different types of membranes with a nominal pore diameter of 0.2 μm: nitrocellulose (NC) (Whatman Nitrocellulose, GE Healthcare Life Science, Buckinghamshire, UK), polyvinylidenedifluoride (PVDF) (Immuno-Blot PVDF Membrane for Protein Blotting, BioRad, Hercules, USA) and polycarbonate (PC) (Whatman Nuclepore Track-Etched Polycarbonate, GE Healthcare Life Science) were tested. PVDF membranes were activated before starting the assays by submerging the membranes in methanol and washing in distilled water. We also used the commercial filters Millex® Filter Unit (Merck Millipore Corporation, Burlington, USA) as filters to sterilize solutions.

Different blocking reagents were tested to reduce the unspecific binding antibody-membrane. We compared two protein blocking reagents: Western Blocking Reagent (WBR) (Roche Life Science, Basel, Switzerland) and Bovine Sero albumin (BSA) (Sigma-Aldrich Co., St Louis, MO, USA) at concentrations of 0.5%, 1% and 2.5% and two detergent blocking reagents: Tween-20 (Tw-20) (Sigma-Aldrich) and Triton 100 (TX-100) (Sigma-Aldrich) at concentrations of 0.1%, 0.25% and 1%. All blocking solutions were prepared using phosphate-buffered saline (PBS) 0.01M (Sigma-Aldrich) as the solution-base.

3.2.2. Bacterial samples and cultivation

An environmental *L. pneumophila* Sg 1 isolate was grown on buffered charcoal yeast extract culture plates (BCYE, Oxoid; Thermo Fisher Scientific, Waltham, MA, USA) for 4 days at 37 °C.

3.2.3. Initial proposal

The initial proposal was divided into 5 steps: membrane blocking, *L. pneumophila* sample filtration, antibody incubation, washes and reaction development (Figure 3.1). All measurements were performed in triplicate, and averages and standard deviations were calculated.

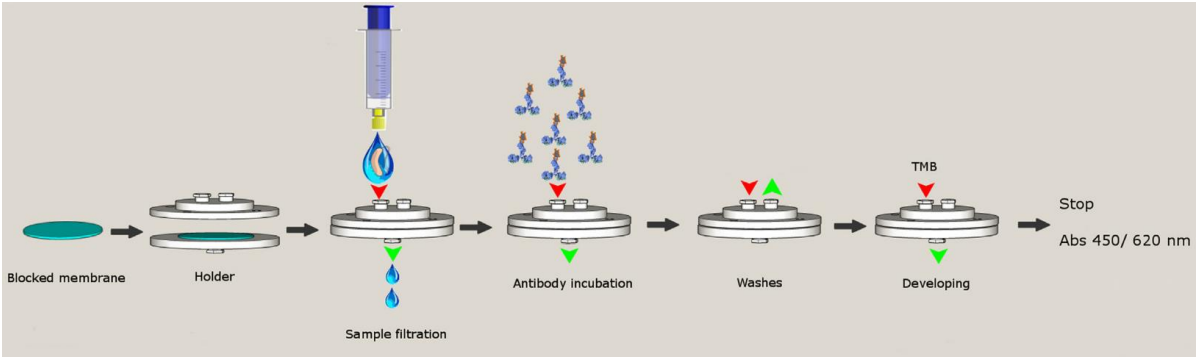

Figure 3.1. Initial proposal of the process of sample filtration and immunological detection of *L. pneumophila*.

3.2.4. Retention test

We tested the *L. pneumophila* retention rate of the different membrane discs with a diameter of 25 mm. Fifty mL of distilled water inoculated with 10^2,10^4 and 10^6 CFU were filtered and seeded directly on the culture plates to quantify the bacterial load retained in the membranes. The CFU obtained with each membrane were compared with the results obtained using the commercial filters.

3.2.5. Blocking step

The blocking capacity of four different treatments was evaluated by comparing the differences between treated and untreated membranes. The membranes (NC, PVDF, and PC) were manually cut into 5mm diameter discs and were treated with different blocking solutions. The membrane discs were incubated for 2 h at 10 rpm in a rotor (Orbit, J.P Selecta, Abrera, Spain) in 1.5 mL tubes containing 500 µL of each blocking reagent (WBR, BSA, Tw-20, TX-100) at different concentrations (0.5%, 1% and 2.5% for proteins and 0.1%, 0.25% and 1% for detergents). The blocking capacity was tested by comparing unspecific antibody binding (See Section 3.2.7).

3.2.6. Blocking optimization

The quantification of blocking loss was calculated for Tw-20 by comparing blocked-unfiltered membranes and blocked-filtered membranes. Twenty-five mm diameter membranes of NC, PVDF and PC were used, and 2 concentrations of Tw-20 solution

(0.1% and 1%) were tested as blocking solution and 0.5% Tw-20 as washing solution. The blocking step was performed by submerging the membranes in 5 mL of each blocking solution for 2 h at 80 rpm using an orbital shaker (POS-300 Grant-bio, Grant Instruments, Cambridge, UK).

Distilled water was filtered through the membranes. Different combinations of distilled water volumes (30, 70, 100 mL) and flow rates (0.1, 0.5, 1 $mL \cdot s^{-1}$) were evaluated in a home-made holder and a peristaltic pump (XX8000230, Merck Millipore). The blocking loss was tested by comparing unspecific antibody binding (See Section 3.2.7).

The blocking step was assessed to establish the appropriate moment to block in the protocol. Membranes were tested without blocking, blocking before filtration and blocking after filtration. The membranes were filtered with distilled water or 10^6 CFU of *L. pneumophilla*. The buffers used were: 1% Tw-20 as blocking buffer and 0.5% Tw-20 as washing buffer. The results obtained in each case were compared to establish the most effective protocol.

3.2.7. Immunological reaction

We used the monoclonal LP3IIG2 (LP3IIG2 ATCC ® HB-8472™) horseradish peroxidase-labeled antibody for the specific detection of *L. pneumophila* [15]. This antibody was obtained from the hybrid cell line HB-8472 (ATCC). This hybridoma was cultured in RPMI Medium 1640+ Glutamax supplemented with 10% of Fetal Bovine serum (both from Gibco) and supernatant was frozen until use. The monoclonal antibody was purified from the supernatant by affinity chromatography using a Hi- Trap Protein G HP column (G&E Healthcare). After dialysis, the monoclonal antibody was conjugated with horseradish peroxidase (HRP) using the Lightning-Link HRP kit (Innova Biosciences) following the manufacturer instructions. Hybridoma supernatant, monoclonal antibody purification and conjugation were made into the SCAC (Cell Culture, Antibody Production and Flow Cytometry Facility of Autonomous University of Barcelona).

The membrane discs were transferred to tubes containing 500 µL of LP3IIG2 antibody-HRP at a final concentration of 0.5 $\mu g \cdot \mu L^{-1}$ in PBS and were incubated for 1 h in rotation. They were then washed 3 times with Tw-20 at concentrations 0.05%, 0.1%, and 0.5% for 5 min in rotation to determine the most efficient washing

concentration. Finally, membranes were transferred to a new tube for a final wash with PBS.

Membranes were placed, using forceps, on the bottom of ELISA 96 Microwell well plates (Nunc-ImmunoMicroWell 96 well, Sigma-Aldrich) and 50 µL (for 5 mm discs) or 150 µL (for 25 mm discs) of the colorimetric substrate 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma-Aldrich) were added and incubated in the dark during 16 min (sufficient time to stabilize the enzyme-substrate reaction, data not shown). The reaction was stopped, adding the same volume of 1 M H_2SO_4, and the substrate reaction was quantified by measuring absorbance at 450 nm in an ELISA reader (Varioskan Flash, ThermoScientific).

3.2.8. Protocol optimization and determination of detection limit

The protocol was optimized to improve the resolution between controls and *L. pneumophila* samples. We tested the effect of a previous fixation step and shaking, incubation times and antibody concentration.

All the optimization process was performed with 25 mm membranes discs and incubations were performed in 6-well plates.

Fixation step: we compared the signal obtained between fixed and unfixed membranes. The fixation step was performed with controls and *L. pneumophila* samples (10^6 CFU) by drying the membrane after filtration.

Shaking: controls and *L. pneumophila* samples (10 CFU) were tested with and without shaking in all incubations. Incubation times: the blocking step was progressively reduced from 2 h to 5 min (2 h, 1 h, 30 min and 5 min), as was antibody incubation (from 1 h to 30 min). The signal obtained at each incubation time was compared to the initial incubation time (2 h for blocking step and 1 h for antibody step) to evaluate the background obtained in controls and the signal obtained in *L. pneumophila* samples (10^6 CFU).

Antibody concentration tested: 0.2, 0.5, 1 and 5 $\mu g \cdot \mu L^{-1}$.

Finally, the detection limit was calculated by filtrating 30 mL of 1/10 serial dilutions of *L. pneumophila* (10^6 CFU) and a control with distilled water following the final protocol of the assay. The detection limit was established using the values obtained

with the controls calculated as the average plus 3 times the standard deviation. This value was used as the cut off of a positive/negative sample.

3.2.9. Integration of the assay protocol into the concentration device:

The assay protocol optimized in the previous section was integrated into the home-made concentration device. Samples of 200 mL of serial dilutions of *L. pneumophila* (10^2-10^5 CFU) and a control with only distilled water were filtered through the 25 mm diameter NC membranes by a peristaltic pump at a flow rate of 0.5 $mL \cdot s^{-1}$. After the sample filtration and fixation step, the reaction chamber of the holder was filled with 1% Tw-20 using a syringe and incubated for 30 min to block the membranes. Next, with another syringe, 700 µL of LP3IIG2 antibody-HRP at a final concentration of 0.5 $\mu g \cdot \mu L^{-1}$ in PBS were injected and incubated for 1h. Finally, by a peristaltic pump at 0.03 $mL \cdot s^{-1}$, 0.5% Tw-20 for 15 min and PBS for 5 min were passed through the holder using the two Luers of the upper part of the device. Between steps, the chamber was emptied.

Once these steps finished, with a syringe, 200 µL of TMB were added and incubated for 16 min. Finally, 100 µL of the reacted TMB were taken out from the holder and placed in a 96-well ELISA plate to stop the TMB reaction with 150 µL of 1M H_2SO_4. Absorbance at 450 nm was read.

3.2.10. Statistical analysis

All measurements were performed in triplicate, and averages and standard deviations were calculated. Statistical tests were performed with GraphPad Prism 7 (GraphPad Software, Inc; La Jolla, USA) to compare quantitative data (t-test). The differences among conditions were defined by a p-value < 0.05.

3.3 RESULTS

3.3.1 Retention test:

The three membranes tested presented the same retention rate as the commercial filters used to sterilize samples. The retention rate established for the three membranes was 100%, since we did not obtain any colony for the culture plate of the seeded filtered volume. These results demonstrated that the three materials were compatible with the filtration of samples containing *L. pneumophila* regardless of the concentration and that the home-made holder was sufficiently watertight to carry out the filtration process.

3.3.2 Blocking test

Figure 3.2 shows the effect of the blocking treatments. On comparing the blocking reagents, we found that the higher the TX-100 concentration the greater the signal, and therefore TX-100 was discarded. We could not conclude whether these values were due to TX-100-TMB interference or because TX-100-treatment promoted unspecific binding of the antibody. The BSA was also discarded for the NC and PVDF membranes because there were no significant differences at most of the concentrations tested in comparison with unblocked membranes (unspecific binding).

The WBR and Tw-20 showed the best blocking performance in the three types of membranes at all the concentrations tested, but unspecific binding was only observed at 0.1% Tw-20 in PVDF.

Washing steps with 0.5% Tw-20 showed significant differences in all three membranes ($p<0.005$) on comparing blocked and unblocked membranes. In most of the cases, the washing concentration was more relevant than the blocking reagent used. In the washing step, the membrane was not only washed but the blocking process was also continued.

On comparing WBR and Tw-20, we selected Tw-20 as the best blocking reagent because of its lower cost, possibility of long-term storage at room temperature and easier preparation.

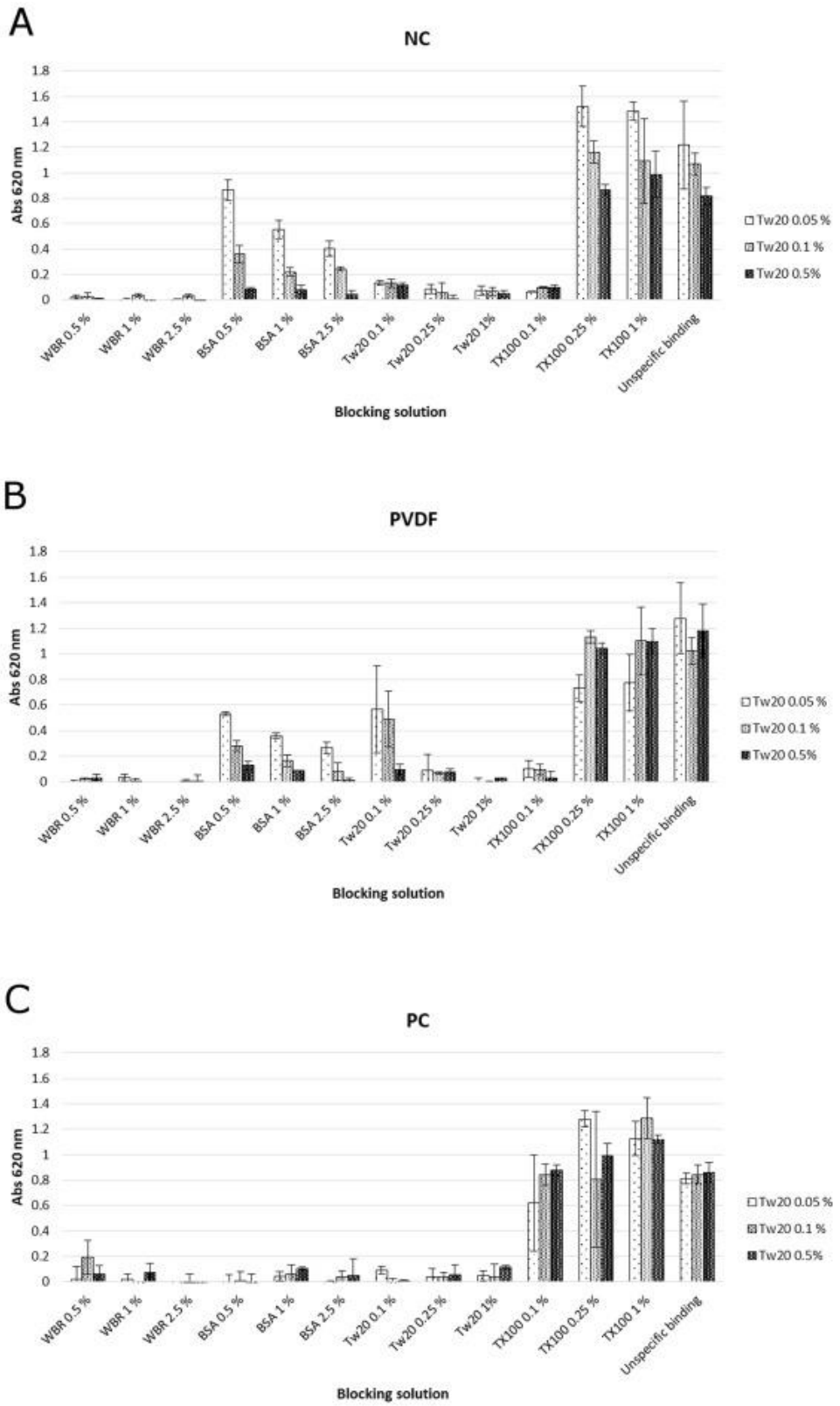

Figure 3.2. Blocking step: unspecific binding antibody-membrane test with membranes: NC (A), PVDF (B) and PC (C), blocking reagents (WBR, BSA, Tw20 and TX100) and different concentrations of washing buffer (0.05%, 0.1% and 0.5% Tw20). The maximum unspecific binding signal is also shown (unspecific binding) as a result of the analysis without any blocking treatment.

3.3.3. Blocking loss analysis

In general, the blocking step before filtration produced heterogeneous results among replicates (Figure 3.3). NC blocked with 1% Tw-20 was the only treatment that did not show this filtration effect. In most of the combinations studied, blocking was not lost, with significant differences between blocked-filtered and unblocked membranes ($p<0.05$). In these cases, the membrane remained blocked after filtration.

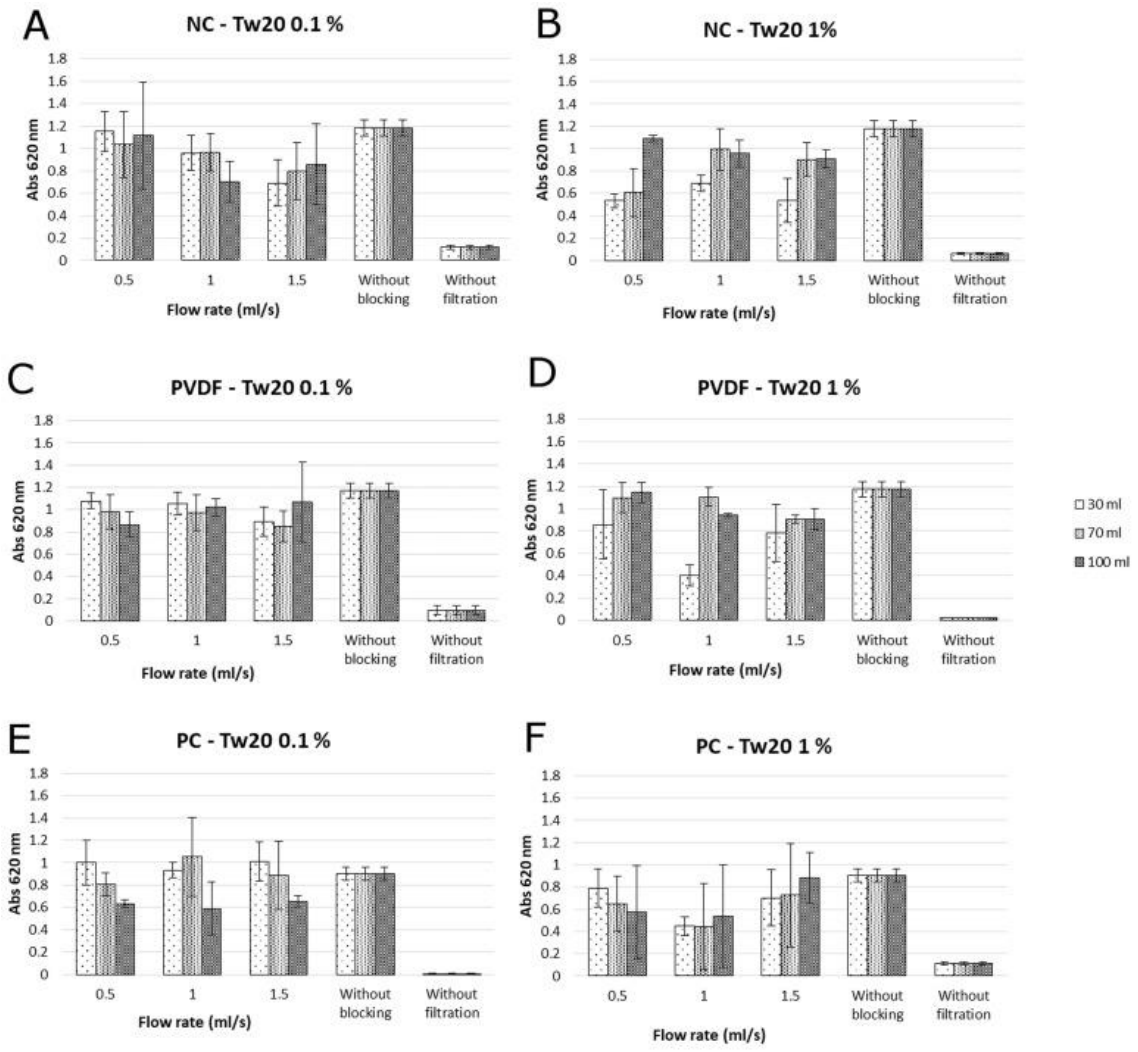

Figure 3.3. Blocking loss test: NC (A and B), PVDF (C and D) and PC (E and F) membranes were blocked (0.1% and 1% Tw-20) and filtration volumes (30, 70 and 100 mL) were filtered through these blocked membranes using different flow rates (0.5, 1 and 1.5 $mL \cdot s^{-1}$). The results obtained for each membrane with each Tw-20 concentration were compared taking into account that unfiltered membranes (without blocking loss) had the lowest values of unspecific antibody binding, and membranes without blocking had the highest values of unspecific antibody binding.

PVDF membranes blocked with 0.1% Tw-20 showed no differences when the volume or flow rate of the filtration changed. When the blocking concentration was increased to 1% Tw-20, the deviations obtained were higher. PC membranes presented the highest variance with high deviations that hampered analysis, thereby providing less reproducible results.

In most cases, the filtration length was more decisive than the flow rate. We observed that by filtering a specific volume, the blocking loss was lower using higher flow rates. The best results in terms of reduction of blocking loss (results closer to those obtained without filtration) were obtained using NC with 1% Tw-20 blocking with the washing solution used (Tw-20 0.5%), 30 mL as a sample volume and 0.5 $mL \cdot s^{-1}$ as the flow rate.

Our first proposal for the protocol was to block the membranes before filtration in order to commercialize the membranes and reduce filter manipulation. However, as the water flow through the membrane affected to this previous step, a blocking after filtration was tested. The blocking step performed after filtration improved the resolution of the assay in NC and PVDF membranes (Figure 3.4), obtaining differences between controls and *L. pneumophila* samples ($p < 0.005$). PC membranes showed worse results in blocking after filtration with a higher signal with controls than with *L. pneumophila* samples. So, it seemed that the nature of the membranes could have influence on the *Legionella* entrapment. PC is a microperforated membrane while NC and PVDF membranes are a fiber web with a specific pore diameter. Cells are retained in PC membranes, but the membrane acts as a strainer, whereas in PVDF and NC membranes the bacteria are immersed in the web. For this reason, when the PC membranes were submitted to incubations, the cells detached from the filter. This characteristic is important in the ISO 11731 method, since the filtrated membrane is resuspended in a small volume of distilled water and then cultured. In contrast in our protocol, cell resuspension during blocking incubation, does not allow them to remain retained in the membrane before the immunodetection process, and as a consequence *Legionella* detection is not possible. So, PC membranes were discarded.

On the other hand, as mentioned above, PVDF and NC were able to show differences between controls and *L. pneumophila* samples: However, the nonspecific binding of the antibody observed for the control was higher for the PVDF membranes. Moreover, these membranes have hydrophobic nature and need to be treated before using them for water filtration. As a result, NC membranes blocked after filtration with Tw-20

were selected for further optimizations to try increasing the resolution of the detection assay. Furthermore, blocking after filtration allowed modification of the sample volume and the flow, which would help to improve the detection limit of the method.

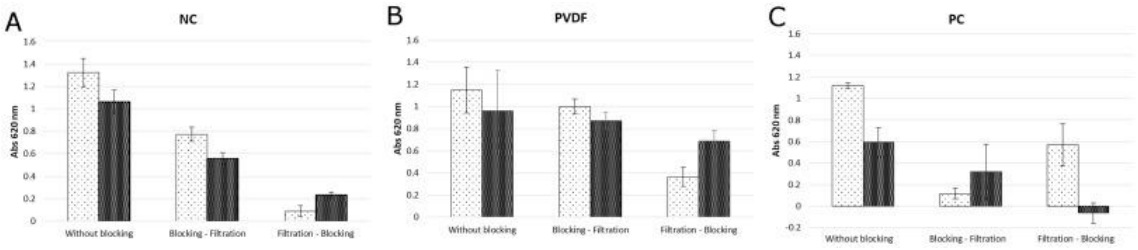

Figure 3.4. Optimization of blocking step: NC (A), PVDF (B) and PC (C) membranes were tested with controls (white columns) and with 10^6 *L. pneumophila* CFU (shaded columns) and were tested without blocking, blocking before filtration and blocking after filtration.

3.3.4. Protocol optimization

The protocol was optimized to improve the resolution between controls and *L. pneumophila* samples. The addition of the fixation step between filtration and blocking substantially increased the resolution between distilled water and *L. pneumophila* samples ($p < 0.05$) (Appendix A, Figure 4). The ELISA signal significantly increased without shaking ($p < 0.05$) (Appendix A, Figure 5). The duration of the protocol was optimized by reducing the blocking and antibody incubation times without losing signal in *L. pneumophila* samples. The blocking incubation time was reduced from 2 h to 30 min (Appendix A, Figure 6A) with no loss of resolution between controls and *L. pneumophila* samples ($p > 0.05$). Using 5 min of blocking, the resolution was reduced to levels of no differences between these two samples. The antibody incubation time was fixed at 1 h ($p<0.005$) (Appendix A, Figure 6B). This incubation time could not be shortened due to a reduction in resolution, obtaining no differences between samples. Lower background and better resolution between controls and *L. pneumophila* samples (Appendix A, Figure 7) were achieved using 0.5 $\mu g \cdot \mu L^{-1}$ of antibody. Although significant differences were obtained between controls and *L. pneumophila* samples at all concentrations, with a final antibody concentration of 0.5 $\mu g \cdot \mu L^{-1}$ the signal obtained in controls was 75% less than the signal obtained with *L. pneumophila* samples.

3.3.5. Assay protocol and detection limit

According to above results, the final protocol (Figure 3.5) was: filtration of sample in 25mm diameter NC filters at a flow rate of 0.5 mL·s^{-1} and fixation by drying 5 min at room temperature. After transfer to a 6-well plate, block membrane 30 min with 2 mL of 1% Tw-20. Incubate for 1 h with 2 mL of LP3IIG2 antibody-HRP at a final concentration of 0.5 µg·µL^{-1} in PBS. After washing the membranes three times with 2 mL of 0.5% Tw-20 and once with PBS, transfer to a new 6-well plate and add 150 µL of TMB. Finally, incubate for 16 min with TMB, stop the reaction with 150 µL of 1M H_2SO_4 and quantify the amount of antibody attached to the membrane by reading the absorbance of the TMB at 450 and 620 nm. Reading at 450 nm always offered a greater differentiation between *Legionella* containing samples and controls (data not shown), so 450 nm was established as the reading absorbance. The detection limit of the optimized assay was established at an Abs_{450nm} of 0.75. The whole protocol , was able to differentiate a distilled water sample from a water sample in less than 2 h showing a detection limit of 70 bacteria in the filter (Figure 3.6).

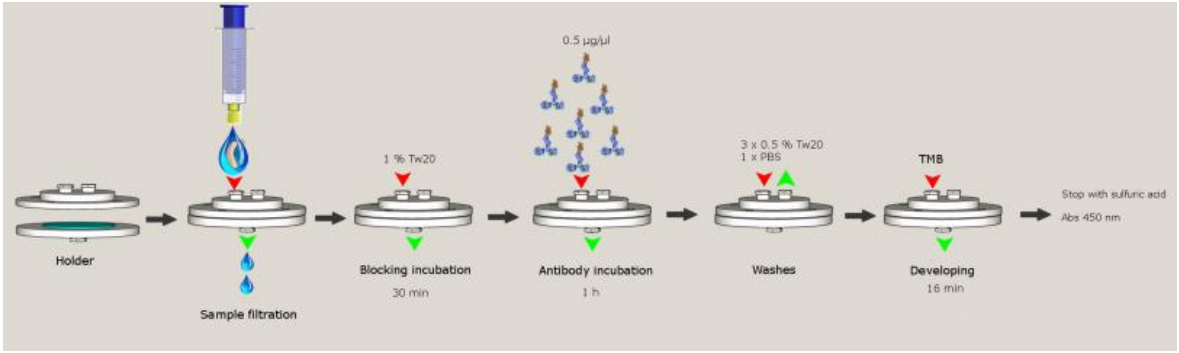

Figure 3.5. Final protocol of the process of sample filtration and immunological detection of *L. pneumophila*.

The assay was able to detect 70 CFU of *L. pneumophila* in a 25 mm filter, regardless of the filtered volume established, in our experiments 200 mL. In this way, our system is capable of filtering volumes of 1 L so that the detection limit that we achieved is within the same order of magnitude as the system approved by the ISO11731 (50 CFU·L^{-1}). This limit of detection is only applicable to in vitro conditions, the conditions under which the tests were performed. To establish the detection limit in natural water samples, which may contain bacteria interfering with *Legionella* and generate background, the protocol has to be tested against natural known positive

and negative water samples for *L. pneumophila*. This new analysis of different types of samples (cooling towers, spas, fountains, condensers) will allow the detection limit fixed in this study to be slightly modified. We have already performed a preliminary test with potable water (data not shown) and the results obtained were equivalent to those obtained with the application of the ISO 11731 method.

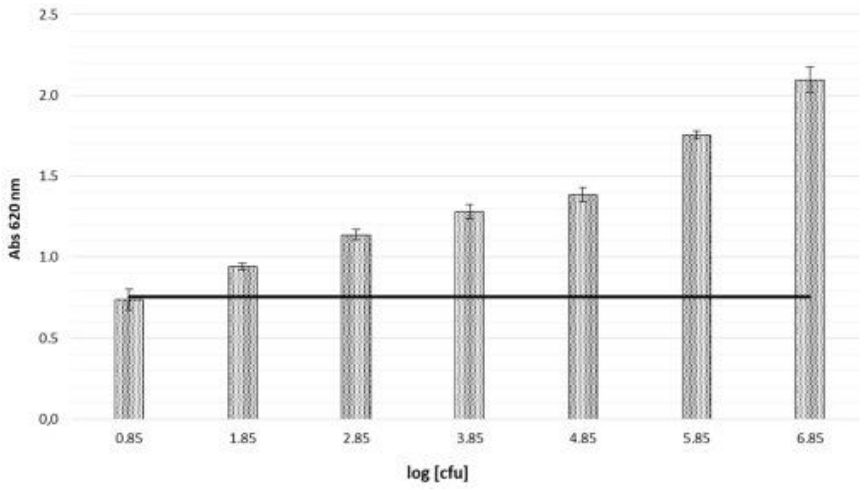

Figure 3.6. Detection limit test: the optimized protocol was applied to serial dilutions of *L. pneumophila* and to MilliQ water samples, used as controls, obtaining a threshold of 0.75 (detection limit is shown as a horizontal line).

The assay was able to detect 70 CFU of *L. pneumophila* in a 25 mm filter, regardless of the filtered volume established, in our experiments 200 mL. In this way, our system is capable of filtering volumes of 1 L so that the detection limit that we achieved is within the same order of magnitude as the system approved by the ISO11731 (50 $CFU \cdot L^{-1}$). This limit of detection is only applicable to in vitro conditions, the conditions under which the tests were performed. To establish the detection limit in natural water samples, which may contain bacteria interfering with *Legionella* and generate background, the protocol has to be tested against natural known positive and negative water samples for *L. pneumophila*. This new analysis of different types of samples (cooling towers, spas, fountains, condensers) will allow the detection limit fixed in this study to be slightly modified. We have already performed a preliminary test with potable water (data not shown) and the results obtained were equivalent to those obtained with the application of the ISO 11731 method.

Nonetheless, it should be taken into account that the samples used were of potable water, which does not have a large amount of microbiota. This favored the differentiation of positive and negative samples, due to a probable reduction in cross-reactivity. A larger number of samples, including other types of samples should be analyzed in order to obtain conclusive results.

3.3.6. Integration of the assay protocol to the concentration platform

Finally, it was demonstrated that the developed assay protocol could be integrated into the home-made concentration holder as the first approach to system automation. As described in a previous study [18], the upper part of the concentration platform is provided with two holes for universal Luer connections and a reaction chamber that facilitates the entrance and exit of the reagents necessary for incubation processes and reagent changes. Thus, in addition to the sample concentration, blocking, antibody incubation, washing, and TMB incubation steps could be carried out inside the holder.

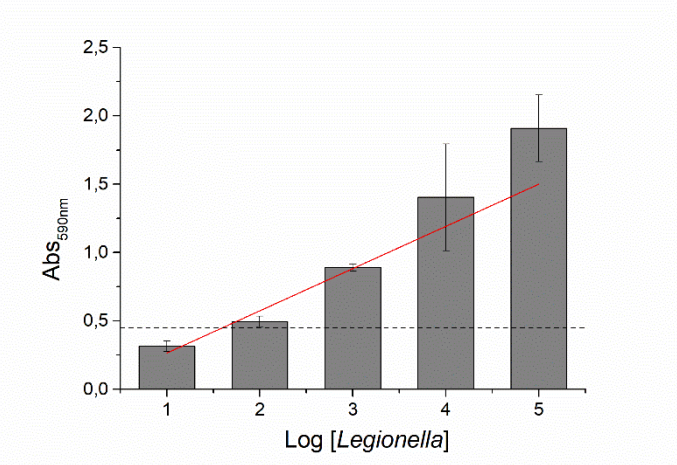

Figure 3.7. Absorbance values obtained for serial dilutions of *L. pneumophila* and controls (MilliQ water samples) after performing the whole protocol inside the home-made concentration holder.

The results demonstrated the possibility to perform the whole assay inside the concentration platform. In this way, different concentrations of *Legionella* could be distinguished (Figure 3.7) with a LoD of 40 CFU in 2h. Comparing these results with the previously in which the detection process was carried out outside the holder (Figure 3.6), the absorbance increase with the increase of *Legionella* concentrations was slightly sharper. Hence, the obtained LoD was practically the same (40 CFU with

respect to 70 CFU). Thus, it could be demonstrated that the developed protocol could be integrated into the concentration platform. Moreover, in future works, the reagent changes could be totally automated using micro-pumps, reducing the hand manipulation errors, and replacing bulky peristaltic pumps. So, a fast and user-friendly device could be achieved.

3.4 CONCLUSIONS

Waterborne diseases affect more than half of the population worldwide, although this situation differs between developing and developed countries. In developing nations, waterborne diseases mainly cause infections of the gastrointestinal tract such as diarrhea [19], while in developed nations, the incidence of these kinds of infections is lower. Indeed, from 2011 to 2012 *Legionella* accounted for 66% of drinking water-associated outbreaks in the United States [20]. These data show the importance of developing a system able to detect *L. pneumophila* colonization of a water system in a fast and specifically.

The on-filter concentration and detection protocol developed in this study, has demonstrated the ability to detect low concentrations of *Legionella* in only 2 h. Moreover, thanks to its capacity to work with water volumes even up to 1 L, the system could be able to match the detection limits stablished by the regulation standards. The protocol has been demonstrated to be simple enough to be automated into an integrated device. Thus, we have developed an easy, rapid and inexpensive system that could be automated as a portable device or installed in a water facilities, and be used by untrained personnel. Moreover, the signal measurement could also be quantified by electrochemical techniques since the reaction of the antibody HRP with the TMB substrate is a redox reaction, searching for more sensitive transduction signals.

Finally, the main advantages of the developed on-filter concentration/detection system is that, choosing an adequate antibody and optimizing the protocol for the capture and detection, this protocol could be easily applied to other target microorganisms.

REFERENCES

[1] ⟨https://ecdc.europa.eu/sites/portal/files/documents/AER_for_2015-legionnaires- disease_0.pdf⟩., (n.d.).

[2] C.B. Fliermans, Ecology of legionella: From data to knowledge with a little wisdom, Microb. Ecol. 32 (1996) 203–228. https://doi.org/10.1007/BF00185888.

[3] M. Sabria, V.L. Yu, Hospital-acquired legionellosis: Solutions for a preventable infection, Lancet Infect. Dis. 2 (2002) 368–373. https://doi.org/10.1016/S1473-3099(02)00291-8.

[4] A.F. Kaufmann, J.E. Mcdade, C.M. Patton, J. V. Bennett, P. Skaliy, J.C. Feeley, D.C. Anderson, M.E. Potter, V.F. Newhouse, M.B. Gregg, P.S. Brachman, Pontiac fever: Isolation of the etiologic agent (Legionella pneumophila) and demonstration of its mode of transmission, Am. J. Epidemiol. 114 (1981) 337–347. https://doi.org/10.1093/oxfordjournals.aje.a113200.

[5] Genus: Legionella, (n.d.). https://www.bacterio.net/genus/legionella (accessed March 20, 2020).

[6] V.L. Yu, J.F. Plouffe, M.C. Pastoris, J.E. Stout, M. Schousboe, A. Widmer, J. Summersgill, T. File, C.M. Heath, D.L. Paterson, A. Chereshsky, Distribution of Legionella Species and Serogroups Isolated by Culture in Patients with Sporadic Community-Acquired Legionellosis: An International Collaborative Survey, J. Infect. Dis. 186 (2002) 127–128. https://doi.org/10.1086/341087.

[7] E.W. Rice, K.R. Fox, H.D. Nash, E.J. Read, A.P. Smith, Comparison of media for recovery of total coliform bacteria from chemically treated water., Appl. Environ. Microbiol. 53 (1987) 1571–1573. https://doi.org/10.1128/aem.53.7.1571-1573.1987.

[8] H. Liu, Y. Yang, J. Cui, L. Liu, H. Liu, G. Hu, Y. Shi, J. Li, Evaluation and implementation of a membrane filter method for Cronobacter detection in drinking water, FEMS Microbiol. Lett. 344 (2013) 60–68. https://doi.org/10.1111/1574-6968.12155.

[9] J.D. Oliver, Recent findings on the viable but nonculturable state in

pathogenic bacteria, FEMS Microbiol. Rev. 34 (2010) 415–425. https://doi.org/10.1111/j.1574-6976.2009.00200.x.

[10] ONDOAN lidera el proyecto LegioPoC para la detección preventiva de la legionela - Ondoan, (n.d.). https://www.ondoan.com/ondoan-lidera-proyecto-legiopoc-la-deteccion-preventiva-la-legionela (accessed March 20, 2020).

[11] Innosieve Diagnostics, ⟨http://www.innosieve.com/project_pinvialeg.html⟩, (n.d.)., (n.d.).

[12] G.R. Albalat, B.B. Broch, M.J. Bono, Y. Chen, Method modification of the legipid® Legionella fast detection test kit: Performance Tested MethodSM 111101, J. AOAC Int. 97 (2014) 1403–1409. https://doi.org/10.5740/jaoacint.14-029.

[13] M.A. Yáñez, C. Carrasco-Serrano, V.M. Barberá, V. Catalán, Quantitative detection of Legionella pneumophila in water samples by immunomagnetic purification and real-time PCR amplification of the dotA gene, Appl. Environ. Microbiol. 71 (2005) 3433–3441. https://doi.org/10.1128/AEM.71.7.3433-3441.2005.

[14] R.A. Deshmukh, K. Joshi, S. Bhand, U. Roy, Recent developments in detection and enumeration of waterborne bacteria: a retrospective minireview, Microbiologyopen. 5 (2016) 901–922. https://doi.org/10.1002/mbo3.383.

[15] N. Párraga-Niño, S. Quero, N. Uria, O. Castillo-Fernandez, J. Jimenez-Ezenarro, F.X. Muñoz, M. Sabrià, M. Garcia-Nuñez, Antibody test for Legionella pneumophila detection, Diagn. Microbiol. Infect. Dis. 90 (2018) 85–89. https://doi.org/10.1016/j.diagmicrobio.2017.11.005.

[16] M. Souiri, N. Blel, D. Sboui, L. Mhamdi, T. Epalle, R. Mzoughi, S. Riffard, A. Othmane, AFM, CLSM and EIS characterization of the immobilization of antibodies on indium-tin oxide electrode and their capture of Legionella pneumophila, Talanta. 118 (2014) 224–230. https://doi.org/10.1016/j.talanta.2013.09.049.

[17] N. Yamaguchi, Y. Tokunaga, S. Goto, Y. Fujii, F. Banno, A. Edagawa, Rapid on-site monitoring of Legionella pneumophila in cooling tower water using a portable microfluidic system, Sci. Rep. 7 (2017) 3092. https://doi.org/10.1038/s41598-017-03293-9.

[18] J.J. Ezenarro, N. Uria, Ó. Castillo-Fernández, N. Párraga, M. Sabrià, F.X. Muñoz Pascual, Development of an integrated method of concentration and immunodetection of bacteria, Anal. Bioanal. Chem. 410 (2018) 105–113. https://doi.org/10.1007/s00216-017-0695-x.

[19] M.P. Miagostovich, F.F.M. Ferreira, F.R. Guimarães, T.M. Fumian, L. Diniz-Mendes, S.L.B. Luz, L.A. Silva, J.P.G. Leite, Molecular detection and characterization of gastroenteritis viruses occurring naturally in the stream waters of Manaus, Central Amazônia, Brazil, Appl. Environ. Microbiol. 74 (2008) 375–382. https://doi.org/10.1128/AEM.00944-07.

[20] Surveillance for waterborne disease outbreaks associated with drinking water - United States, 2011-2012, Am. J. Transplant. 15 (2015) 3260–3267. https://doi.org/10.1111/ajt.13602.

4. ON-FILTER IMMUNOASSAY AND AMPEROMETRIC MEASUREMENTS FOR RAPID DETECTION OF *Legionella pneumophila*

ABSTRACT

Legionella is a pathogenic bacterium, ubiquitous in freshwater environments and able to colonize man-made water systems from which it can be transmitted to humans during outbreaks. The prevention of such outbreaks requires a fast, low cost, automated and often portable detection system. In the present work, we present a device that combines sample concentration, immunoassay detection, and measurement by chronoamperometry. A nitrocellulose microfiltration membrane inside a custom-made holder is used as support for the water sample concentration and *Legionella* immunodetection. The horseradish peroxidase enzymatic label of the antibodies permits using the redox substrate 3,3′,5,5′-Tetramethylbenzidine to generate current changes proportional to the bacterial concentration present. Carbon screen-printed electrodes are employed in the chronoamperometric measurements. Our system reduces the detection time: from the 10 days required by the conventional culture-based methods, to 2-3 h, which could be crucial to avoid outbreaks. Additionally, the sensor shows a linear response (R^2 value of 0.99), being able to detect a range of *Legionella* concentration between 10^1 and 10^5 CFU·mL^{-1} with a detection limit (LoD) of 4 CFU·mL^{-1}.

4.1. INTRODUCTION

Legionella pneumophila is a waterborne pathogen, able to generate outbreaks that can vary in severity from non-pneumonic Pontiac fever (2-5 days illness) to Legionnaires' disease (LD), which fatality rate ranges from 5-30 % or even higher for the elderly, children, and immunosuppressed persons [1–5]. *Legionella pneumophila*, an ubiquitous bacterium present in many freshwater environments, is able to colonize man-made water systems such as showers, cooling towers or whirlpool spas where it can grow unchecked and be transmitted to humans by inhalation or micro-aspiration of aerosols [6–8].

A good surveillance program is required to prevent such events in facilities at risk [9,10]. Currently, the gold standard methods used for *Legionella* detection are based on culture techniques (ISO 11731), which are labor-intensive, time-consuming (10 days), and require laboratory facilities [11,12]. Faster molecular methods such as PCR (polymerase chain reaction) have been developed, but these need highly skilled personnel, specific instrumentation, and are more costly [13]. Out of the 16 serogroups of *Legionella pneumophila*, serogroup 1 causes the majority of the European and American isolates [14]. Some commercial kits have been developed for fast *Legionella* detection. LegionellaFast, from *Legionella* Control International, detects the presence of *Legionella pneumophila* serogroup 1 using an LFICA (Lateral Flow Immunochromatographic Assay). This device gives results within 25 min on-site without the need for special equipment or specialist expertise. However, this system is only able to detect *Legionella* serogroup 1, giving a yes/no result [15]. Legipid® *Legionella* Fast Detection from Biotica is a test that combines sample concentration by filtration and magnetic immunocapture with an enzyme-immunoassay (CEIA) for the colorimetric detection of *Legionella* in water with a low limit of detection of 40 CFU. Nevertheless, the assay is carried out manually and needs to be performed by qualified personnel [16].

Over the past 20 years, biosensors have emerged as an attractive alternative for pathogen detection, since they are easy to miniaturize and automate while providing faster analysis times. Biosensors use biological recognition mechanisms to provide measurable quantitative or semi-quantitative information. A biorecognition element (e.g., enzyme, antibodies, nucleic acids, aptamer, cell receptors, and phages) binds the target of interest and a transducer (optical, electrochemical, mass-based, thermometrical or micromechanical) converts this event into a measurable signal

[17–19]. Nevertheless, the vast majority of the biosensors developed for *Legionella* detection still have high detection limits and need sample pre-concentration steps prior to analysis.

Herein, a *Legionella pneumophila* detection device, in which a nitrocellulose microfiltration membrane acts as the support for the sample concentration as well as for the antigen-antibody reaction (Figure 4.1). The anti-*Legionella* antibody employed for the biorecognition is labeled with HRP enzyme that with the help of mediators such as 3,3′,5,5′-Tetramethylbenzidine (TMB), makes possible to observe current changes proportional to the concentration of target in the samples [20,21]. The system is able to detect *Legionella* concentrations on the rage of 10^1-10^5 CFU·mL^{-1} with a low limit of detection (LoD) of 4 CFU·mL^{-1}. Additionally, the whole process of concentration, immunoassay and chonoamperometric measurement takes only 2h.

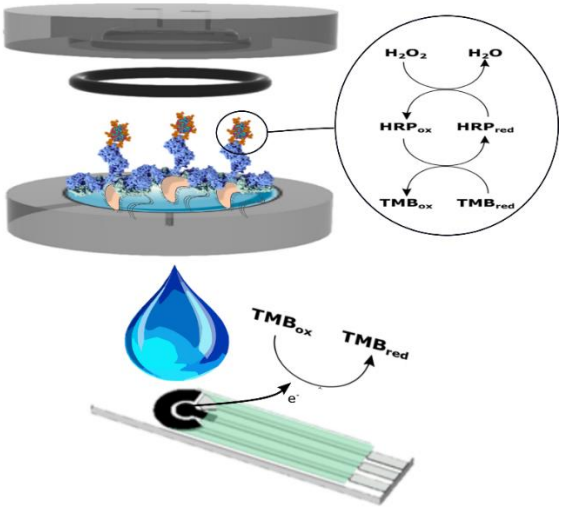

Figure 4.1. General scheme. The membrane retains the *Legionella* cells for the subsequent immunoassay and the chronoamperometric transduction of the signal.

4.2. MATERIALS AND METHODS

4.2.1 Microorganisms and growth conditions

Legionella pneumophila Sg1 isolated from environmental samples was grown on buffered charcoal yeast extract culture plates (BCYE, Oxoid; Thermo Fisher

Scientific, Waltham, MA, USA) for 4 days at 37 °C. Colonies from grown plates were scraped and resuspended in sterile water. Bacterial suspensions were standardized to an OD_{625nm} of 0.3 and further diluted to provide samples with a final concentration of 10^1 to 10^5 CFU·mL^{-1}.

4.2.2 Electrode fabrication and electrochemical characterization

Chip layout was designed using Vectorworks 2016 (Techlimits, ES) and the electrodes were screen-printed using a home-made manual press, using 25x25 cm/20x20 cm (outer dimensions/inner dimensions) screens meshed at 90 threads/cm, and using shore 75 square polyurethane squeegees. The snap-off distance was 0.5 mm for conducting inks, and 1 mm for the dielectric coating. Carbon paste C2030519P4 (Gwent Electronics materials Ltd, UK) was used for printing the working and auxiliary electrodes. Silver paste Electrodag 725A (Henkel, ES) was used to print the pseudo-reference electrodes, tracks, and contact pads. A layer of UV curable dielectric Electrodag PF-455B (Henkel, ES) was used to protect the conducting tracks between the contact pads and the electrodes and define the electrode area. These electrodes were screen printed directly on a 0.5 mm thick polyethylene terephthalate (PET) substrate (Autostat, MacDermid, UK).

This design consisted of a central 2.5 mm diameter working electrode graphite disc surrounded by a graphite auxiliary electrode and a silver pseudo-reference electrode.

Electrochemical measurements were performed to characterize the electrode behavior and reproducibility. These measurements were carried out with a Palm Sense4 potentiostat (PalmSense BV) controlled by a PC running PSTrace 5.4 software. As redox substrate, a ready to use commercial preparation of 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma-Aldrich) containing hydrogen peroxide (H_2O_2) was used (composition no provided by the supplier). Six different electrodes were used to test the reactivity of the TMB at the electrode and analyze the reproducibility of the response. To this end, 100 µL of TMB were deposited on the electrodes and cyclic voltammetry was carried out at potentials between -200 mV and +600 mV at a scan rate of 50 mV. Averages and standard deviations of the current and potentials of the oxidation-reduction peaks were calculated.

4.2.3 *Legionella pneumophila* concentration and antibody reaction

Different inoculums between 10^1 and 10^5 CFU·mL^{-1} in a final volume of 200 mL of water were filtered through 25 mm diameter nitrocellulose (NC) membrane (Whatman Nitrocellulose, GE HealthcareLife Science) with a nominal pore of 0.2 µm at a flow rate of 0.5 mL·s^{-1} by a peristaltic pump (XX8000230, Merck Millipore). Similarly, 200 mL of water without bacterial cells were filtered as blank. After filtration, membranes were transferred to a 6-well plate and air-dried for 5 min. Next, membranes were incubated in 2 mL of 1% v/v Tween-20 (Sigma-Aldrich) in 0.01 M phosphate buffered saline (PBS, Sigma-Aldrich) for 30 min. After that, the samples were treated with 2 mL of LP3IIG2 anti-*legionella* antibody-HRP at a final concentration of 0.5 µg·µL^{-1} in PBS for 1h. After this, the membranes were washed three times with 2 mL of 0.5% Tween-20 in PBS and once with only PBS solution for 5 min. Subsequently, the membranes were transferred to a new 6-well plate and 150 µL of TMB was added. A reaction time of 16 min between the HRP enzyme and TMB substrate was defined. In previous experiments, it was observed that 16 min were sufficient to obtain a stable signal.

The antibody was selected from a previous work [22] as a specific sensitive antibody for the detection of *L. pneumophila*. This study confirmed that the LP3IIG2 antibody was able to recognize almost all the serogroups of *L. pneumophila* and did not cross-react with other microbial species.

The holder employed to perform the filtrations either the immunoassay carried out at this study is described more in detail in previous works [14,23].

4.2.4 Electrochemical measurements for *Legionella* detection

Following the 16 min immunodetection reaction, 100 µL of TMB were deposited on the electrode and the current was measured at 50 mV vs. Ag (determined from the cyclic voltammetry performed to characterize the electrodes) for 240 s (time enough to stabilize the current signal). Finally, we compared the current values at different times during the chronoamperometry (10, 25, 50, 100 and 240 s) for the different *Legionella* concentrations. All measurements were carried out by triplicate and the averages and standard errors were calculated. The limit of detection of the system was calculated as the bacterial concentration equal or higher than the current signal value calculated as the blank current + 3 times the blank standard deviation.

4.3 RESULTS AND DISCUSSION

4.3.1 Electrode and redox substrate characterization

The fabricated screen-printed electrodes were analyzed by cyclic voltammetry (CV) using TMB, the chromogenic and redox substrate employed later in the *Legionella* detection protocol (Figure 4.2). TMB is one of the most used substrates, as its oxidation mechanism is well-known [24] and it offers the possibility of performing either optical or electrochemical measurements.

TMB undergoes a two-electron oxidation-reduction process [24,25]. We confirmed this by cyclic voltammetry using our electrodes (Figure 4.2A). Two oxidation peaks at 180 mV and 350 mV (vs Ag) (red dashed arrows) and two reduction peaks at 250 mV and 100 mV (vs Ag) (blue dotted arrows), respectively, were observed. Moreover, CVs were performed using six different screen printed electrodes (SPE) to test the reproducibility. Good reproducibility of the electrodes was observed with a variability of 3% and 7% for the current intensity and the potential of the peaks, respectively.

TMB reaction with anti-*Legionella* antibody labeled with HRP enzyme was also characterized with our electrodes. When HRP was involved on the redox reaction of TMB (Figure 4.2B), the CV continued showing two oxidation (250 mV and 440 mV vs Ag) and 2 reduction peaks (350 mV and 200 mV vs Ag). However, compared to the CV observed when HRP is not involved at the reaction (Figure 4.2B), the peak potentials shifted to higher potentials and the measured peak currents were lower. This is attributed to the action of the H_2O_2 present in the enzymatic substrate solution, which can oxidize the Ag pseudo-reference electrode but also passivate the working electrode. Figure 4.2B inset demonstrates that although the HRP enzyme oxidizes the TMB substrate, TMB is actually reduced at the electrode. Based on the voltammetric responses shown in Figures 4.2A and 4.2B, a working potential of 50 mV vs. Ag was selected. Besides, the current background at the selected potential is near zero, indicating that no substrate oxidation occurs. These conditions are suitable for measuring low amounts of product in the presence of high substrate concentrations [26,27].

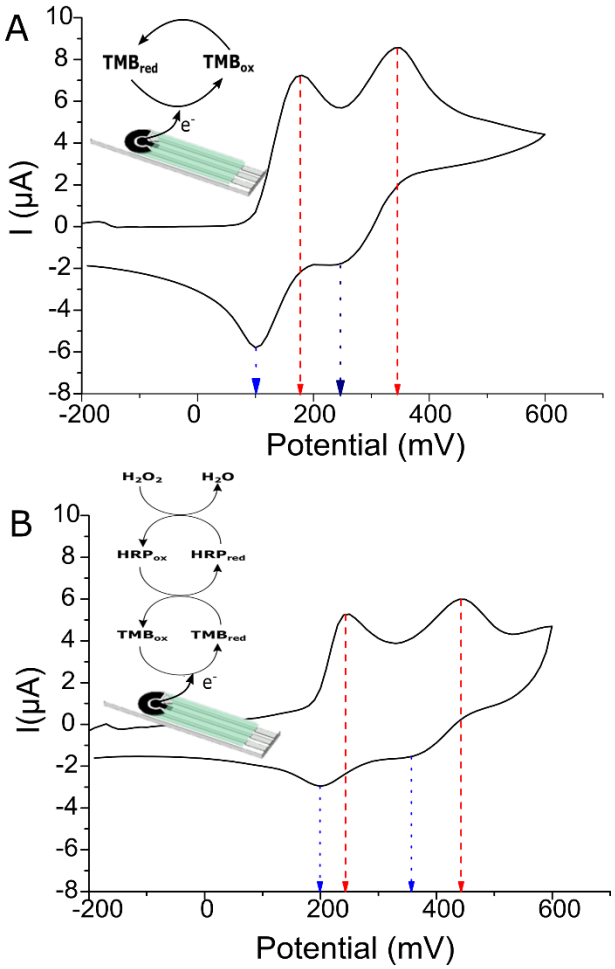

Figure 4.2. A) Cyclic voltammetry of the substrate 3,3',5,5'-Tetramethylbenzidine (TMB) in a SPE. B) Cyclic voltammetry of the substrate 3,3',5,5'-Tetramethylbenzidine (TMB) and horseradish peroxidase (HRP) reaction. Peak potentials are highlighted in red (oxidation) and blue (reduction).

In case of the measurement of an ideal blank (without *Legionella*), no HRP-labelled antibody should be present in the electrode and CV very similar to the one depicted in Figure 4.2A should be observed, confirming the suitability of a polarization potential of 50 mV (vs Ag).

4.3.2 Development of a calibration curve for the detection and quantification of *Legionella*

To test the capacity of the system to detect *Legionella pneumophila* in water, 200 mL samples containing different *Legionella* concentrations ranging between 10^1-10^5 CFU·mL^{-1} were filtered. Then, once immunoassay and incubation with the TMB

substrate were carried out, chronoamperometric measurements were carried out at a reduction potential of 50 mV for a total of 240 seconds (Figure 4.3).

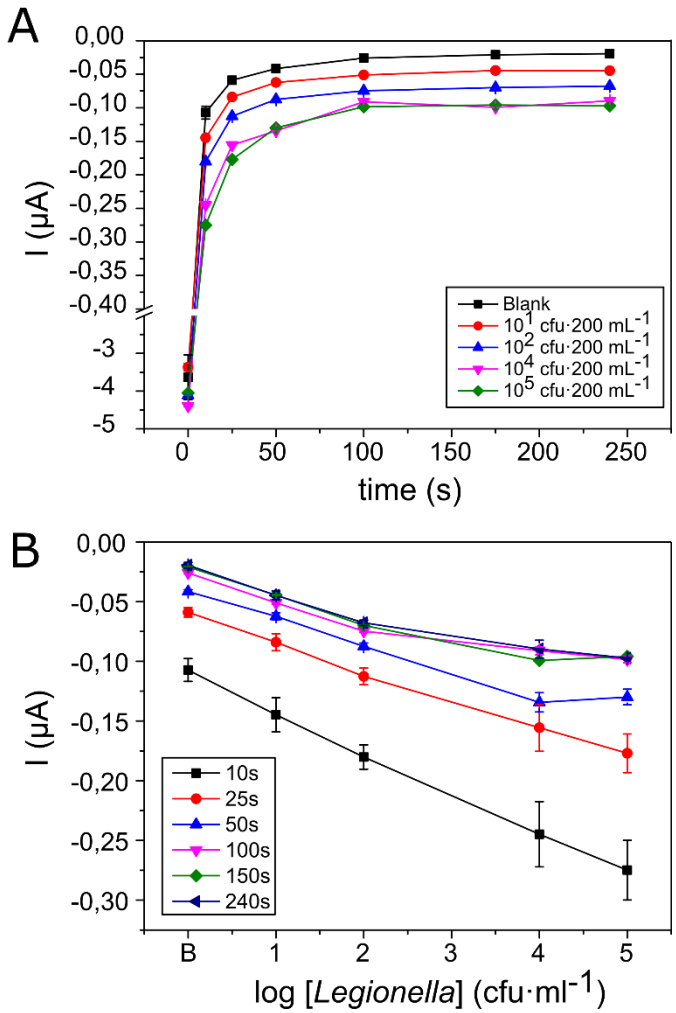

Figure 4.3. A) Current change response in time, measured for different *Legionella* concentrations. B) Current changes measured for different *Legionella* concentrations at different times (from 0 to 240 s). Standard error bars correspond to the measurements made in three different culture replicates of each concentration measured in two different assays (n=6).

Figure 4.3A shows that after a fast current increase during the first 50 seconds resulting from electrode polarization, the reaction at the electrode stabilized and started to reach a plateau state. Additionally, it was observed that differences in the current values obtained by the different *Legionella* concentrations were lower, particularly among 10^4 and 10^5 CFU mL^{-1}, the highest concentrations analyzed. This is clearly observed in Figure 3.B, where current values were represented in relation

to *Legionella* concentration at different reaction times. The results showed a linear relationship between *Legionella* concentration and the current obtained in short measurement periods between 10 and 25 s. However, after 50 s of reaction, linearity was lost at the highest concentration of *Legionella,* probably as a consequence of the passivation of the electrodes related to TMB precipitation. When HRP oxidizes TMB, the resultant blue product deposits on the electrode surface, blocking it and reducing the current obtained [28,29]. At high bacterial concentrations, TMB is oxidized faster and therefore, the passivation effect is observed sooner. In addition to linearity loss, this could also explain the large standard deviations found at high bacterial concentrations.

Finally, despite the loss of linearity at the highest bacterial concentration (10^5 CFU·mL^{-1}), we decided to use the readings taken at time 50 s because they provided an equilibrium between good dynamic range (10^1 to 10^4 CFU·mL^{-1}) and low variability.

In Figure 4.4 the absolute values of stable current recorded at time 50 s has been plotted as a function of the concentration of *Legionella pneumophila* present in the samples.

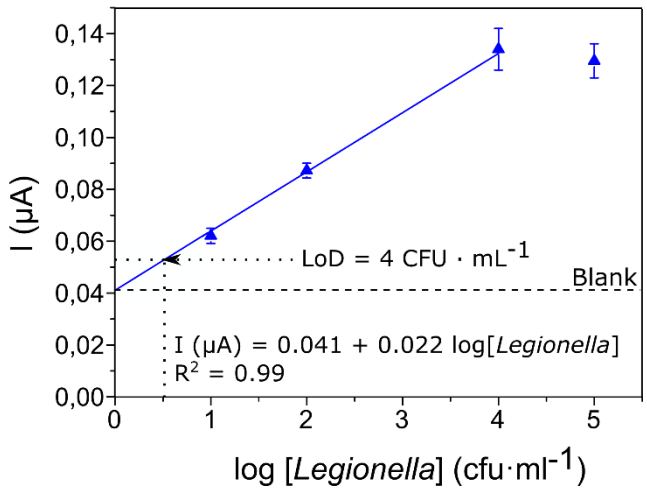

Figure 4.4. Sensor calibration curve where the current obtained at a constant potential of 50 mV at the second 50 of the chronoamperometry is expressed in absolute values as a function of the logarithm of the increasing concentrations of *Legionella* from 0 (blank) to 10^5 CFU·mL^{-1}. The regression line is indicated in blue and error bars represent the standard error (n=6).

A regression line was fitted to the values (R^2 = 0.99), excluding the last point at which TMB precipitation on top of the electrodes had caused passivation and unreliable readings. The resolution of the curve fitting indicated a sensitivity (expressed as the slope of the I vs log[*Legionella*] curve) of 22 nA/log[*Legionella*] in the range of 10^1 and 10^4 CFU·mL^{-1}.

Additionally, the detection limit of our system was determined taking into account the variability of the chronoamperometric current obtained in blank samples (samples without *Legionella*). This limit was established as a current value of 0.053 µA corresponding to a *Legionella* concentration of about 4 cells per milliliter. We concluded that the proposed method can detect the presence of *Legionella* at low concentrations but without any of the disadvantages of current standard methods. .

Currently ISO 11731:2017 and ISO/TS 12869:2019 are by far the most used methods for isolation and estimation of *Legionella* in water. On one hand, ISO 11731:2017 is a culture based method where a concentration by membrane filtration is needed for the detection of less than 10^4 CFU·L^{-1} ("ISO 11731:201"). Nevertheless, despite its high sensitivity and ability to comply with the 10^2-10^3 CFU·L^{-1} standard set by most regulatory agencies (World Health Organization, 2007), it requires highly skilled personnel and takes 7-10 days to provide results. On the other hand, ISO/TS 12869:2019 which is based on quantitative polymerase chain reaction (qPCR) ("ISO 12869:2019"), is faster and simpler than ISO 11731:2017 and provides similar sensitivity. However, it needs sophisticated instrumentation and qualified personnel.

Methods based on biosensors are gaining attention due to the advantages they offer in terms of miniaturization and automation, providing fast and user-friendly detection devices. Notwithstanding, currently available biosensors lag behind in terms of sensitivity and in most cases are unable to match the requirements of the current regulatory frameworks.

Genosensors detect DNA, RNA or PCR amplified products that come from the target cells. These methods express their detection limits as concentration of nucleic acids, without stating clearly the equivalence to actual *Legionella* concentrations. Thus, although quantitative methods detecting nucleic acids, as the ISO 12869:2019 exist, these biosensors provide qualitative results. [34–38]. Moreover, steps of nucleic acid extraction and concentration are difficult to automatize and require expensive equipment and qualified personnel.

Immunosensors for *Legionella* are generally based on sandwich immunoassays in which a first antibody immobilized on the sensor captures the target, while a secondary labelled antibody transduces the bio-recognition into a measurable signal. Nevertheless, the use of a two-antibody system makes the analysis more expensive. Moreover, they have high detection limits in the range of 10^6-10^8 CFU·L^{-1} [39–43] and, the one able to achieve a lower detection limit (10^4 CFU·L^{-1}) needed a pre-concentration step and even so it did not reach the standards established for *Legionella* surveillance [40].

The system we present in this work integrates concentration and immunoassay by the use of a microfiltration membrane as a support for both. Thus, the sample concentration process is already included in the device's own operation. To carry out the whole process (concentration, as well as the different steps of the immunoassay and the chronoamperometric measurement) takes 2-3h to provide reliable results. This may seem long compared to the 30 to 45 min reported by some genosensors [35–37], but these claims do not take into account the time needed for sample concentration preparation. Additionally, as cells are retained in the membrane due to the filtration step, there is no need for a capture antibody. Thus, a single antibody system is used for the immunoassay lowering the cost for each test.

4.4 CONCLUSIONS

Effective *Legionella* monitoring and surveillance in freshwater systems is essential in order to avoid outbreak appearance. In this work, we have developed an electrochemical immunoassay system that provides the advantage of utilizing a microfiltration membrane that acts as the support for sample concentration and immunodetection, giving the chance to treat big sample volumes and increase the possibility to detect low *Legionella* amounts with a detection limit of 4 CFU·mL^{-1}.

Additionally, the integration of the concentration and immunodetection steps into a single holder allows obtaining a simpler and faster system that gives results within 2h. Thus, the developed detection system is able to overcome one of the biggest drawbacks of the gold standard method ISO 11731, the detection time.

As a result, we have accomplished the objective of obtaining a rapid, economical and user-friendly sensor for *Legionella pneumophilla* detection: Moreover, was specially designed in such a way that in future versions all steps can be automated and carried out by micropumps without the need for qualified personnel and fabricated with low-cost materials that could easily be mass-produced.

REFERENCES

[1] J.E. McDade, Legionella and the Prevention of Legionellosis , Emerg. Infect. Dis. 14 (2009) 1006a – 1006. https://doi.org/10.3201/eid1406.080345.

[2] M. Yoshida, N. Furuya, N. Hosokawa, H. Kanamori, M. Kaku, M. Koide, F. Higa, J. Fujita, Legionella pneumophila contamination of hospital dishwashers, Am. J. Infect. Control. 46 (2018) 943–945. https://doi.org/10.1016/j.ajic.2018.01.024.

[3] W. Dana Flanders, K.H. Kirkland, B.G. Shelton, Effects of holding time and measurement error on culturing Legionella in environmental water samples, Water Res. 62 (2014) 293–301. https://doi.org/10.1016/j.watres.2014.05.025.

[4] R.M. Atlas, Legionella: From environmental habitats to disease pathology, detection and control, Environ. Microbiol. 1 (1999) 283–293. https://doi.org/10.1046/j.1462-2920.1999.00046.x.

[5] H. Whiley, Legionella risk management and control in potable water systems: Argument for the abolishment of routine testing, Int. J. Environ. Res. Public Health. 14 (2017). https://doi.org/10.3390/ijerph14010012.

[6] C. Caicedo, K.H. Rosenwinkel, M. Exner, W. Verstraete, R. Suchenwirth, P. Hartemann, R. Nogueira, Legionella occurrence in municipal and industrial wastewater treatment plants and risks of reclaimed wastewater reuse: Review, Water Res. 149 (2019) 21–34. https://doi.org/10.1016/j.watres.2018.10.080.

[7] H. Tronel, P. Hartemann, Overview of diagnostic and detection methods for legionellosis and Legionella spp., Lett. Appl. Microbiol. 48 (2009) 653–656. https://doi.org/10.1111/j.1472-765X.2009.02570.x.

[8] E. Jubete, O.A. Loaiza, E. Ochoteco, J.A. Pomposo, H. Grande, J. Rodríguez, Nanotechnology: A Tool for Improved Performance on Electrochemical Screen-Printed (Bio)Sensors, J. Sensors. 2009 (2009) 1–13. https://doi.org/10.1155/2009/842575.

[9] I. Torre, R. Alfano, T. Borriello, O. De Giglio, C. Iervolino, M.T. Montagna, M.S. Scamardo, F. Pennino, Environmental surveillance and in vitro activity of antimicrobial agents against Legionella pneumophila isolated from hospital

water systems in Campania, South Italy: a 5-year study, Environ. Res. 164 (2018) 574–579. https://doi.org/10.1016/j.envres.2018.02.030.

[10] D. Sboui, M. Souiri, S. Reynaud, S. Palle, M. Ben Ismail, T. Epalle, R. Mzoughi, F. Girardot, S. Allegra, S. Riffard, A. Othmane, Characterisation of electrochemical immunosensor for detection of viable not-culturable forms of Legionellla pneumophila in water samples, Chem. Pap. 69 (2015) 1402–1410. https://doi.org/10.1515/chempap-2015-0170.

[11] R. Petrisek, J. Hall, Evaluation of a most probable number method for the enumeration of Legionella pneumophila from North American potable and nonpotable water samples, J. Water Health. 16 (2018) 57–69. https://doi.org/10.2166/wh.2017.118.

[12] L. Jaber, M. Amro, H.A. Tair, S.A. Bahader, H. Alalam, S. Butmeh, D.A. Hilal, I. Brettar, M.G. Höfle, D.M. Bitar, Comparison of in situ sequence type analysis of Legionella pneumophila in respiratory tract secretions and environmental samples of a hospital in East Jerusalem, Epidemiol. Infect. 146 (2018) 2116–2121. https://doi.org/10.1017/s0950268818002340.

[13] Z. Altintas, M. Akgun, G. Kokturk, Y. Uludag, A fully automated microfluidic-based electrochemical sensor for real-time bacteria detection, Biosens. Bioelectron. 100 (2018) 541–548. https://doi.org/10.1016/j.bios.2017.09.046.

[14] N. Párraga-Niño, S. Quero, A. Ventós-Alfonso, N. Uria, O. Castillo-Fernandez, J.J. Ezenarro, F.X. Muñoz, M. Garcia-Nuñez, M. Sabrià, New system for the detection of Legionella pneumophila in water samples, Talanta. 189 (2018) 324–331. https://doi.org/10.1016/j.talanta.2018.07.013.

[15] Rapid Self-Test for Legionella - LegionellaFast, (n.d.). https://legionellacontrol.com/rapid-self-test-for-legionella-legionellafast/ (accessed March 11, 2019).

[16] G.R. Albalat, B.B. Broch, M.J. Bono, Method Modification of the Legipid ® Legionella Fast Detection Test Kit, J. AOAC Int. 97 (2014) 1403–1409. https://doi.org/10.5740/jaoacint.14-029.

[17] F. Ramírez-Castillo, A. Loera-Muro, M. Jacques, P. Garneau, F. Avelar-González, J. Harel, A. Guerrero-Barrera, Waterborne Pathogens: Detection Methods and Challenges, Pathogens. 4 (2015) 307–334.

https://doi.org/10.3390/pathogens4020307.

[18] H. Bridle, M. Desmulliez, Biosensors for the Detection of Waterborne Pathogens, in: Waterborne Pathog. Detect. Methods Appl., Academic Press, 2013: pp. 189–229. https://doi.org/10.1016/B978-0-444-59543-0.00002-5.

[19] J.T. Connelly, A.J. Baeumner, Biosensors for the detection of waterborne pathogens, Anal. Bioanal. Chem. 402 (2012) 117–127. https://doi.org/10.1007/s00216-011-5407-3.

[20] G. Coussot, C. Faye, A. Le Postollec, M. Dobrijevic, One-step direct immunoassay with horseradish peroxidase as antigen for studying the functionality of antibody surfaces, Talanta. 178 (2018) 922–927. https://doi.org/10.1016/j.talanta.2017.10.032.

[21] A. Ahmed, J. V. Rushworth, N.A. Hirst, P.A. Millner, Biosensors for whole-cell bacterial detection, Clin. Microbiol. Rev. 27 (2014) 631–646. https://doi.org/10.1128/CMR.00120-13.

[22] N. Párraga-Niño, S. Quero, N. Uria, O. Castillo-Fernandez, J. Jimenez-Ezenarro, F.X. Muñoz, M. Sabrià, M. Garcia-Nuñez, Antibody test for Legionella pneumophila detection, Diagn. Microbiol. Infect. Dis. 90 (2018) 85–89. https://doi.org/10.1016/j.diagmicrobio.2017.11.005.

[23] J.J. Ezenarro, N. Uria, Ó. Castillo-Fernández, N. Párraga, M. Sabrià, F.X. Muñoz Pascual, Development of an integrated method of concentration and immunodetection of bacteria, Anal. Bioanal. Chem. 410 (2018) 105–113. https://doi.org/10.1007/s00216-017-0695-x.

[24] P. Fanjul-Bolado, M.B. González-García, A. Costa-García, Amperometric detection in TMB/HRP-based assays, in: Anal. Bioanal. Chem., 2005: pp. 297–302. https://doi.org/10.1007/s00216-005-3084-9.

[25] L.A. Marquez, H.B. Dunford, Mechanism of the oxidation of 3,5,3',5'-tetramethylbenzidine by myeloperoxidase determined by transient- and steady-state kinetics, Biochemistry. 36 (1997) 9349–9355. https://doi.org/10.1021/bi970595j.

[26] E. Baldrich, F.J. del Campo, F.X. Muñoz, Biosensing at disk microelectrode arrays. Inter-electrode functionalisation allows formatting into miniaturised sensing platforms of enhanced sensitivity, Biosens. Bioelectron. 25 (2009) 920–

926. https://doi.org/10.1016/j.bios.2009.09.009.

[27] I. Superiore, V.R. Elena, G. Volpe, R. Draisci, G. Palleschi, D. Compagnone, 3,3′,5,5′-Tetramethylbenzidine as electrochemical substrate for horseradish peroxidase based enzyme immunoassays. A comparative study, Analyst. 123 (1998) 1303–1307. https://doi.org/10.1039/a800255j.

[28] M. Liu, Y. Zhang, Y. Chen, Q. Xie, S. Yao, EQCM and in situ FTIR spectroelectrochemistry study on the electrochemical oxidation of TMB and the effect of large-sized anions, J. Electroanal. Chem. 622 (2008) 184–192. https://doi.org/10.1016/j.jelechem.2008.06.002.

[29] S.D. Kim, J.W. Chung, J.T. Kim, H. Krause, J.C. Pyun, Gold-film array-electrode for electrochemical ELISA, in: Sensors Actuators, B Chem., Elsevier, 2005: pp. 463–469. https://doi.org/10.1016/j.snb.2005.03.057.

[30] ISO 11731:2017(en), Water quality — Enumeration of Legionella, (n.d.). https://www.iso.org/obp/ui/#iso:std:iso:11731:ed-2:v1:en (accessed September 4, 2019).

[31] World Health Organization, Legionella and the Prevention of Legionellosis, 2007. https://www.who.int/water_sanitation_health/emerging/legionella.pdf (accessed September 4, 2019).

[32] BOE.es - Documento BOE-A-2003-14408, (n.d.). https://www.boe.es/buscar/doc.php?id=BOE-A-2003-14408 (accessed September 4, 2019).

[33] ISO - ISO/TS 12869:2019 - Water quality -- Detection and quantification of Legionella spp. and/or Legionella pneumophila by concentration and genic amplification by quantitative polymerase chain reaction (qPCR), (n.d.). https://www.iso.org/standard/70756.html (accessed September 4, 2019).

[34] R. Miranda-Castro, P. De-Los-Santos-Álvarez, M.J. Lobo-Castañón, A.J. Miranda-Ordieres, P. Tuñón-Blanco, Hairpin-DNA probe for enzyme-amplified electrochemical detection of Legionella pneumophila, Anal. Chem. 79 (2007) 4050–4055. https://doi.org/10.1021/ac062260q.

[35] V. Rai, Y.T. Nyine, H.C. Hapuarachchi, H.M. Yap, L.C. Ng, C.S. Toh, Electrochemically amplified molecular beacon biosensor for ultrasensitive DNA sequence-specific detection of Legionella sp., Biosens. Bioelectron. 32 (2012)

133–140. https://doi.org/10.1016/j.bios.2011.11.046.

[36] V. Rai, J. Deng, C.S. Toh, Electrochemical nanoporous alumina membrane-based label-free DNA biosensor for the detection of Legionella sp, Talanta. 98 (2012) 112–117. https://doi.org/10.1016/j.talanta.2012.06.055.

[37] A.M. Foudeh, D. Brassard, M. Tabrizian, T. Veres, Rapid and multiplex detection of Legionella's RNA using digital microfluidics, Lab Chip. 15 (2015) 1609–1618. https://doi.org/10.1039/c4lc01468e.

[38] A.M. Foudeh, J.T. Daoud, S.P. Faucher, T. Veres, M. Tabrizian, Sub-femtomole detection of 16s rRNA from Legionella pneumophila using surface plasmon resonance imaging, Biosens. Bioelectron. 52 (2014) 129–135. https://doi.org/10.1016/j.bios.2013.08.032.

[39] K.F. Lei, P.H.M. Leung, Microelectrode array biosensor for the detection of Legionella pneumophila, Microelectron. Eng. 91 (2012) 174–177. https://doi.org/10.1016/j.mee.2011.10.002.

[40] M. Martín, P. Salazar, C. Jiménez, M. Lecuona, M.J. Ramos, J. Ode, J. Alcoba, R. Roche, R. Villalonga, S. Campuzano, J.M. Pingarrón, J.L. González-Mora, Rapid Legionella pneumophila determination based on a disposable core-shell Fe<inf>3</inf>O<inf>4</inf>@poly(dopamine) magnetic nanoparticles immunoplatform, Anal. Chim. Acta. 887 (2015) 51–58. https://doi.org/10.1016/j.aca.2015.05.048.

[41] A. Meneghello, A. Sonato, G. Ruffato, G. Zacco, F. Romanato, A novel high sensitive surface plasmon resonance Legionella pneumophila sensing platform, Sensors Actuators, B Chem. 250 (2017) 351–355. https://doi.org/10.1016/j.snb.2017.04.177.

[42] B.K. Oh, Y.K. Kim, W. Lee, Y.M. Bae, W.H. Lee, J.W. Choi, Immunosensor for detection of Legionella pneumophila using surface plasmon resonance, in: Biosens. Bioelectron., Elsevier, 2003: pp. 605–611. https://doi.org/10.1016/S0956-5663(03)00032-0.

[43] H.Y. Lin, Y.C. Tsao, W.H. Tsai, Y.W. Yang, T.R. Yan, B.C. Sheu, Development and application of side-polished fiber immunosensor based on surface plasmon resonance for the detection of Legionella pneumophila with halogens light and 850 nm-LED, Sensors Actuators, A Phys. 138 (2007) 299–305. https://doi.org/10.1016/j.sna.2007.05.015.

5. PHOTONIC DEVICE FOR EARLY WARNING OF CYANOBACTERIAL BLOOMS

ABSTRACT

Cyanobacterial blooms produce hazardous toxins, deplete oxygen, and secrete compounds that confer undesirable organoleptic properties to water. To prevent bloom appearance, the World Health Organization has established an Alert Level between 500 and 2000 cells·mL^{-1}, beyond the capabilities of most optical sensors detecting the cyanobacteria fluorescent pigments. Flow cytometry, cell culturing and microscopy may reach these detection limits, but they involve both bulky and expensive laboratory equipment or long and tedious protocols. Thus, no current technology allows fast, sensitive and *in situ* detection of cyanobacteria. Here, we present a simple, user-friendly, low-cost and portable photonic system for in situ detection of low cyanobacterial concentrations in water samples. The system integrates high-performance pre-concentration elements and optical components for fluorescence measurement of specific cyanobacterial pigments, i.e. phycocyanin. Phycocyanin has demonstrated to be more selective to cyanobacteria than other pigments, such as chlorophyll-a, and to present an excellent linear correlation with bacterial concentration from 10^2 to 10^4 cell·mL^{-1} (R^2=0.99). Additionally, the high performance of the pre-concentration system leads to detection limits below 435 cells·mL^{-1} under 10 minutes in aquaponics water samples. Due to its simplicity, compactness and sensitivity, we envision the current technology as a powerful tool for early warning and detection of low pathogen concentrations in water samples.

5.1 INTRODUCTION

Cyanobacteria are oxygenic photoautotroph microorganisms forming part of the phytoplankton assemblages naturally found in freshwater, brackish and marine ecosystems [1–7]. Natural eutrophication of waters (generally nitrogen and phosphorous over-enrichment) and favorable environmental conditions (temperature, light intensity and pH) can induce an uncontrolled cyanobacteria growth, leading to harmful algal bloom (HAB) apparition [8–12].

Cyanobacterial blooms represent a health threat due to the production of a wide range of allergenic, toxic and carcinogenic substances [4,11,13–16], as well as oxygen consumption, which causes aquatic animal death by hypoxia or anoxia [3,6]. Moreover, some of the secreted compounds modify the taste and odour of the water, affecting its quality and that of any product obtained from it, e.g. foodstuff from aquaculture [17,18].

The increasing anthropogenic eutrophication of waters due to wastewater discharge, the intense agricultural and industrial activity and global warming aggravate the frequency and duration of cyanobacterial blooms [17,19–23], which is a problem for the economy and the environment [24]. The World Health Organization (WHO) is well aware of this problem. In an attempt to control and minimize the effect of such blooms, the WHO has established a control protocol considering three alert levels based on cell concentration [25]: (1) Vigilance level, between 500 and 2000 cells·mL^{-1}, corresponding to the possible early stage of a bloom development; (2) Alert Level 1, between 2000 and 100000 cells·mL^{-1}, when the cyanobacteria biomass can produce significant cyanotoxin concentrations that affect water quality; and (3) Alert Level 2, once cyanobacteria concentration exceeds 100000 cells·mL^{-1}, and toxin production represents an actual risk for human health.

Conventional methods for detection and quantification of cyanobacteria include taxonomic analysis and cell counting by microscopy, flow cytometry, polymerase chain reaction (PCR) [26], and Chlorophyll-a (Chl-a) extraction and quantification through optical methods. Unfortunately, taxonomic analysis and pigment extraction rely on tedious and time-consuming protocols requiring trained personnel [20,27,28] and, although much faster, flow cytometry and PCR are expensive technologies involving bulky benchtop equipment impossible to operate outside the laboratory (*in situ*).

Direct optical detection of fluorescent pigments, without the need for extraction, has been recently applied to cyanobacteria/algae detection and demonstrated high sensitivity, specificity, simplicity and the possibility for real-time analysis [29,30]. Although initially limited by the size and cost of optical components [29], inexpensive light-emitting diodes (LEDs) with low power requirements [31,32] have enabled the development of optical systems for direct determination of Chl-a *in situ* and in real-time [33]. Zeng and Li published a review about different *in situ* Chl-a fluorescence sensors analyzing their advantages and disadvantages [34]. The biggest drawback of using Chl-a fluorescence for cyanobacteria is the lack of selectivity of this pigment. Chl-a is present in all phytoplankton species, making it unsuitable for distinguishing between cyanobacteria and microalgae. Additionally, cyanobacteria present low fluorescence magnitudes at the excitation wavelengths of Chl-a and, therefore, the contribution of cyanobacteria to this measurement may be small or none at all [32,35].

Due to the low specificity of Chl-a, alternative fluorescent pigments have been proposed as selective cyanobacteria indicators. Phycocyanin (PhC) has been the first option due to its high specificity to cyanobacteria (it is only present in this and few other species) [36,37]. Phycocyanin is excited between 590 nm and 630 nm, and emits between 650 nm and 660 nm [33,38]. This is sufficiently different from Chl-a [20,39,40] to allow selective detection. Thus, PhC allows the detection of cyanobacteria even in mixed phytoplankton assemblages [5,33]. Based on the later, several PhC fluorescence-based sensors have already been developed, such as the submersible field probes YSI 6600 and TriOS [1,20,41], which can analyze cyanobacteria at different depth levels, or sensor systems with multiple fluorescence channels for more precise spectroscopic analysis [29,32,42]. Despite their selectivity even in real samples, the main drawback of all these systems is their limit of detection, typically above 10^3 cell·mL^{-1}, where the initial stage of the bloom already takes place.

This work presents an integrated photonic system combining high-performance pre-concentration elements with miniaturized fluorescence sensing optical components for sensitive detection of cyanobacteria *in situ*. The system has been used to assess cyanobacteria concentration in water samples and compared to standard Chl-a extraction protocols. Finally, the cyanobacteria sensor has been validated with real samples from fish growth tanks of an aquaponic system.

5.2 MATERIALS AND METHODS

5.2.1 Cyanobacteria culture, sample preparation and quantification

Synechocystis sp. PCC 6803 was used as model cyanobacteria. Cyanobacteria were cultivated at 18°C in BG-11 medium (Sigma-Aldrich) containing trace metal mix A5 (Sigma-Aldrich) under dark:light cycles (12:12 h) at 6000 Lux. Before experiments, several dilutions were prepared in the range from 10^{-1} to 10^{-5} cells mL^{-1} in distilled water. Each dilution was prepared in triplicate.

The concentration of the initial culture of *Synechocystis* sp. was determined by fluorescence microscopy in a Zeiss Imager M2 microscope by the Breeds counting equation (Equation. 5.1) [43].

$$Cell \cdot mL^{-1} = \frac{n°cells \times (analised\ area/microscope\ field)}{volume \times dilution} \qquad \text{Equation 5.1.}$$

where $n°\ cells$ is the average of cell numbers at ten different fields of the holder, *analyzed area* is the area where the sample is spread over, *microscope field* is the area observed under the microscope (40X = 0.08 mm^2), *volume* is the sample volume placed in the holder and *dilution* indicates the dilution of the initial culture.

5.2.2 Chlorophyll-a extraction and quantification

Dilutions of *Synechocystis* sp. PCC 6803 were prepared in a final volume of 1 mL deionized water and centrifuged at 13000 rpm for 5 min (Centrifuge 5804 R, Eppendorf). The cell pellet was re-suspended in 5 mL of absolute methanol (Sigma-Aldrich) and incubated in the darkness at 4˚C for 24h for the Chl-a extraction. After incubation, the total Chl-a content was determined by absorbance spectroscopy at a wavelength of 665 nm using a spectrophotometer (SmartSpec™ Plus, BioRad). The absorbance of the absolute methanol was used as the reference blank.

The obtained absorbance values were related to a Chl-a concentration by the Lambert-Beer equation (Equation. 5.2):

$$A = \varepsilon\, l\, c \qquad \text{Equation 5.2.}$$

where ε is the extinction coefficient that in the case of Chl-a in absolute methanol is 74.5 ml·mg^{-1} ·cm^{-1},[44] l is the optical path length and c is the concentration of chlorophyll-a.

5.2.3 Sample concentration and fluorescence measurement by a plate reader

One-milliliter samples of different concentrations of *Synechocystis* sp. were inoculated in a final volume of 15 mL of distilled water. Afterwards, these samples were filtered through 25 mm diameter nitrocellulose (NC) membranes (Whatman Nitrocellulose, GE Healthcare Life Science) with a nominal pore size of 0.2 μm for cell retention and pre-concentration in the filter. Filtration was carried out using a custom-made pre-concentration holder [45].

After filtration, membranes were transferred to a 6-well plate to perform fluorescence measurements using a Reader Varioskan Flash (Thermo Fisher). An optical filter at a wavelength of 590 nm was used for selective excitation of the cyanobacterial pigment phycocyanin. Fluorescence emission was measured at 660 nm. All measurements were carried out by triplicate, and the averages and standard deviations were calculated.

Finally, a calibration curve was obtained by representing the intensity of the fluorescence peak emission at 665 nm in Relative Fluorescence Units (RFU) versus cyanobacterial concentration (*Synechocystis* cells mL^{-1}). Thus, the values in the linear range were used to determine the linear regression, the limit of detection (LoD) and the limit of quantification (LoQ) using the following equations:

$$LoD = \frac{3 \times SDa}{b} \qquad \text{Equation 5.3.} \qquad LoQ = \frac{10 \times SDa}{b} \qquad \text{Equation 5.4.}$$

Where SDa is the standard error of the response and b is the slope.

5.2.4 Integrated device for in situ *Synechocystis sp.* detection

5.2.4.1 Sensor design and fabrication

A 3D model of the pre-concentration *holder* was designed based on a previous work [45] using VectorWorks 2018 (Techlimits, ES) and machined in polyurethane wood (Necuron 840, NECUMER, DE) using a Roland MDX-40A desktop milling machine. The design included all elements for fluid management and for positioning the optical fibres required for fluorescence measurement in situ. The holder consisted of two parts assembled by screws and sealed by a rubber O-ring (20 mm of inner diameter and 3 mm in width) to ensure water tightness (Figure 5.1B). These parts contained (i) 4 mm thread bolts for universal Luer connectors used as fluidic inlet and an outlet; (ii) a planar socket with evacuation channels to place the nitrocellulose (NC) membrane used for cell retention and pre-concentration; (iii) a cavity of 17.5 mm diameter and 2.8 mm of depth with two holes of 1 mm at 45˚ angle relative to the *z*-axis for the incorporation of the flexible optical fibres (Keyence, ToughFlex (613 core) fibres); and (iv) a second cavity to implement the rubber O-ring. The remaining optical elements were implemented in a second polymeric structure, fabricated in poly-methylmethacrylate (PMMA) by laser ablation (using an Epilog Mini 24), and assembled to the previous one with pressure-sensitive adhesive (PSA).

This PMMA structure was divided into two to simplify assemblage. The first one was attached permanently to the top layer of the holder with double-sided PSA and sealed with single-sided PSA. It included: (i) a hole for the insertion of the Luer used to inject the water sample; (ii) two apertures corresponding to the protruding end of each fiber optics integrated in the top part of the holder; and (iii) two openings for the insertion of 2 mm dowel pins used for the fast and straightforward alignment of the flexible fibre optics incorporated in the holder with the excitation LED and the SMA-plug connection. These two elements were incorporated in the second PMMA structure. This second PMMA structure, therefore, contained (i) a cavity for the suitable and permanent positioning of a LUXEON Rebel “amber” LumiLED light-emitting diode (LED) with an emission peak at 590 nm used as excitation light source; and (ii) a plug connection [46] for an SMA-connected optical fiber (QP600-1-XSR, Ocean Optics, Largo, FL, USA) of 600 µm/1000 µm core/cladding diameter, which was connected to an external spectrometer (QEPro; Ocean Optics, Largo, FL, USA) used as optical detector; and (iii) two holes matching in size and depth with the dowel pins (2 mm) implemented in the first PMMA piece. These holes/pins allowed a simple

assembly between both PMMA pieces while ensuring the alignment of the optical components.

Optical components for fluorescence measurements were controlled and synchronized through a custom printed circuit board (PCB) incorporating a Raspberry Pi microprocessor and implementing a custom-made Python interface, which is available online (Figure 5.1D) [47].

5.2.4.2 Integrated photonic sensor operation for *Synechocystis* quantification

A NC membrane (Amersham™ Protran™, GE Healthcare) was placed inside the *holder* for bacterial pre-concentration. A total volume of 15 mL was filtered through the *holder* at a constant flow rate of 0.5 mL s^{-1} by a peristaltic pump (Master Flex, 7518-10). After filtration, the Luer-lock located at the upper side of the *holder* was replaced by the PMMA piece integrating the LED and the detector, which aligned with the integrated optical fibers thanks to the two dowel pins implemented in the PMMA structure.

Fluorescence measurements of the photosynthetic pigment phycocyanin were taken from the central filter area (~ 1 mm^2) to estimate the *Synechocystis* concentration present in the water sample. Emission spectra for each sample were obtained after subtraction of the dark spectrum obtained when the LED was switched OFF. Therefore, the recording of one spectrum (N = 1) required a measurement cycle comprised of a period of signal acquisition without excitation (dark spectrum acquisition) followed by a second period of identical duration to register the emission of the sample after excitation. For each sample analyzed, 6 dark/excitation cycles were performed, and an average spectrum was obtained and represented. The total duration of the fluorescence measurement was 30 seconds.

5.2.4.3 Measurement of water samples from aquaponic systems

Water samples were provided by the Experimental Fish Farm (PEIMA) of the French National Institute for Agricultural Research (INRA), located in Monts d'Arrée. These samples were taken from three points of the aquaponic system, namely: (i) the Inflow Dam (ID), which corresponded to freshwater from a lake introduced to the aquaponics station; (ii) Outflow Fish (OF), water flowing out from the fish tanks after

mechanic filtration; and (iii) water from the Final Settele (FS), where waters from the whole system converged and were finally collected.

Aliquots of these samples were analysed with the integrated photonic system and by a reference laboratory (LABOCEA, Ploufragan, France) for comparison. The reference laboratory followed standard protocols (golden standard) based on taxonomic analysis and cell counting by microscopy.

5.3 RESULTS AND DISCUSSION

5.3.1 Integrated photonic system for in situ concentration and detection of cyanobacteria

For a field application, a sensing platform was developed for bacterial pre-concentration and in situ detection of cyanobacteria. The platform, illustrated in Figure 5.1B, consisted of a polymeric holder integrating elements for cell pre-concentration and fluorescent measurement, an external spectrometer used as an optical detector, and a custom electronic circuit based on a Raspberry Pi and controlled through a Python application. The design of the holder resembled one already published by the group [45] but, this time, integrating two optical fibers at 45˚angle from the vertical and optical components for in situ fluorescent detection of cyanobacteria. The fibers were positioned in each side of the water entrance forming between them a 90˚ angle that converges at the central surface point of the lower part. This geometry was selected since maximized the efficiency of the emission collection and reduced the interference of the background excitation light [48].

In terms of operation (Figure 5.1C), NC membranes were incorporated in the holder for bacterial retention and pre-concentration. After water filtering, retained cyanobacteria could be detected in situ after excitation of the specific photosynthetic pigment phycocyanin with the amber LED (emission wavelength = 590 nm) and broadband analysis of the emitted light with the external spectrometer connected to the output optical fiber. Excitation pulses for the optical measurement were triggered using a custom PCB (Figure 5.1D) along with the simultaneous data acquisition by the spectrometer. Both were automated via a Raspberry Pi and a custom Python

interface, so the measurements could be done easily and without requiring manual manipulation.

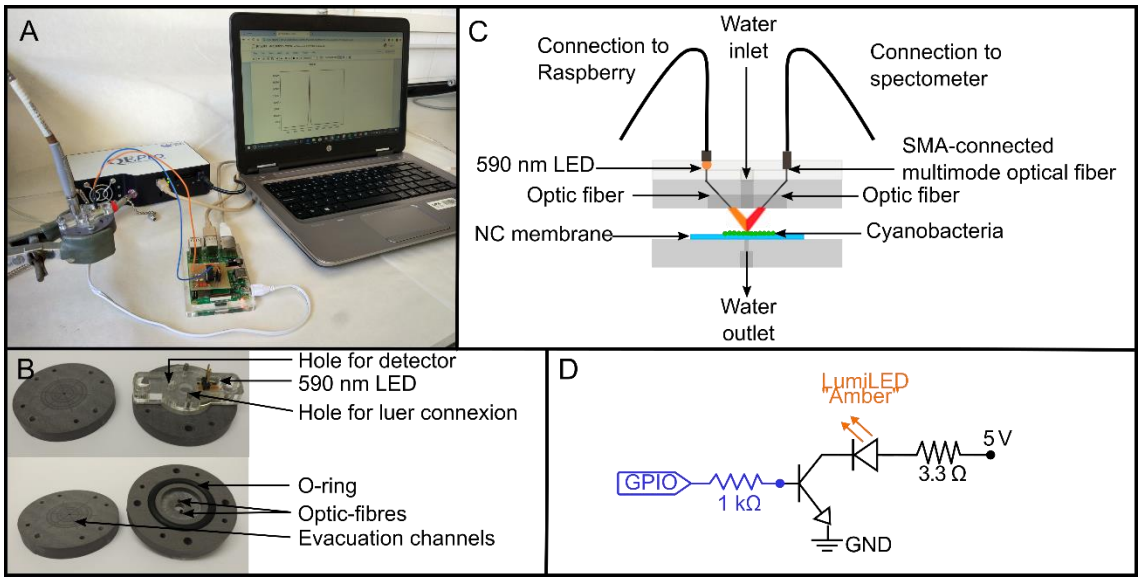

Figure 5.1. A) Fluorescence sensor set-up. B) Polyurethane holder for sample concentration with fiber optics aligned to the LED and the detector. C) Schematic representation of the holder and the sensor operation mode. D) Schematic representation of the circuit mounted on the RPi for the control of the LED through a pin (GPIO).

5.3.2 Evaluation of the performance of the pre-concentration holder in the cyanobacterial analysis based on PhC detection

A first assay was conducted to evaluate the capacity of the pre-concentration holder to retain cyanobacteria and to validate the measurement of PhC in the specific detection of cyanobacteria without extraction. Previous studies demonstrate the correlation between PhC fluorescence and cyanobacteria concentration in recreational water as well as in drinking water, without the need for sophisticated manipulation [33,49]. However, the reported methods fail to reach the concentration levels required by Alert level 1, established by the World Health Organization (WHO). On the other hand, we already demonstrated the filtration capacity of the pre-concentration holder in the retention of *E.coli* and *Legionella* [45,50], being able to concentrate samples of hundreds of milliliters in a few minutes.

To demonstrate the capacity of the filtration holder to retain cyanobacteria, *Synechocystis* suspensions containing bacterial concentrations between 10^1 and 10^6

cells·mL^{-1} were passed through our system. The fluorescent emission of bacterial cells retained on the nitrocellulose filter was determined off-chip using laboratory equipment after excitation at 590 nm. Figure 5.2 shows the relation between cyanobacteria concentration and PhC fluorescence at 660 nm, selected according to bibliography [33,38]. The response was found to be linear in a wide concentration range from 10^1 to 10^4 cells mL^{-1} (Figure 5.2, inset, red line), after which it plateaus out. Low detection and quantification limits of 185 cells·mL^{-1} (LoD) and 616 cells·mL^{-1} (LoQ) were obtained, respectively (Table 5.1). This demonstrates the pre-concentration capacity of the system. Additionally, it is worth noting that the membrane did not present auto-fluorescence in the absence of cyanobacteria, and that the background signal was minimal (0.00±0.03 RFU).

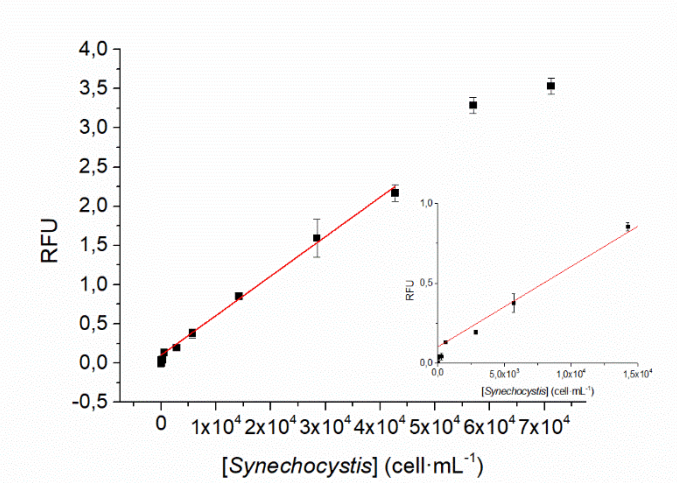

Figure 5.2. Calibration curve representing the relative fluorescence units (RFU) emitted 660 nm by different Synechocystis concentrations after the excitation at 590 nm. The linear regression was calculated for the linear range ($y = 0.102 + 5.08x10^{-5}x$, $R^2=0.99$) and depicted in red. Error bars represent the standard deviation (n=3).

Pre-concentration in the holder and PhC detection by fluorescence was compared to the standard method of cyanobacterial quantification based on Chl-a extraction. As previously stated, Chl-a is a photosynthetic pigment extensively used in the indirect measurement of overall phytoplankton biomass [25,51]. Chl-a may be extracted using different organic solvents, including methanol, acetone or dimethyl sulfoxide [37,52]. After extraction, the concentration of Chl-a can be estimated or quantified by spectrophotometry, high-performance liquid chromatography (HPLC) or fluorimetry, among others. We used absolute methanol extraction and

spectrophotometry for the determination of Chl-a content and cyanobacterial quantification. Figure 5.3 illustrates the correlation between the *Synechocystis* concentration, estimated by microscopy, and the absorbance values at 665 nm corresponding to Chl-a content. As shown, it was a clear linear correlation between Chl-a and *Synochocystis* concentration in the range from 6.3 x 10^4 cell·mL^{-1} to 5.1 x 10^5 cell·mL^{-1} (Red line Figure 5.3). No significant differences were obtained in the fluorescence magnitude below 10^4 cells·mL^{-1}, suggesting that this method was not sensitive enough to detect low bacterial concentrations. The LoD and LoQ were determined in this case, providing values of 1.3 x 10^4 cells·mL^{-1} and 4.3 x 10^4 cells·mL^{-1} respectively (Table 1), between 2 and 3 orders of magnitude higher than PC fluorescence after pre-concentration with the holder, and with a much larger variation coefficient (3% for PC fluorescence detection versus 18% for Chl-a) due to the variability of the extraction protocol. Additionally, cyanobacteria quantification by Chl-a extraction is a tedious, complex and long procedure taking about 24 hours, skilled personnel, and expensive lab equipment.

Therefore, the procedure reported here, which involves bacterial pre-concentration followed by PhC fluorescent detection was superior to standard Chl-a extraction methods in terms of simplicity, cost, time-to-result, sensitivity and specificity. Next, we discuss how the incorporation of the sensing/transduction elements would allow in situ determination of cyanobacteria in an integrated photonic system.

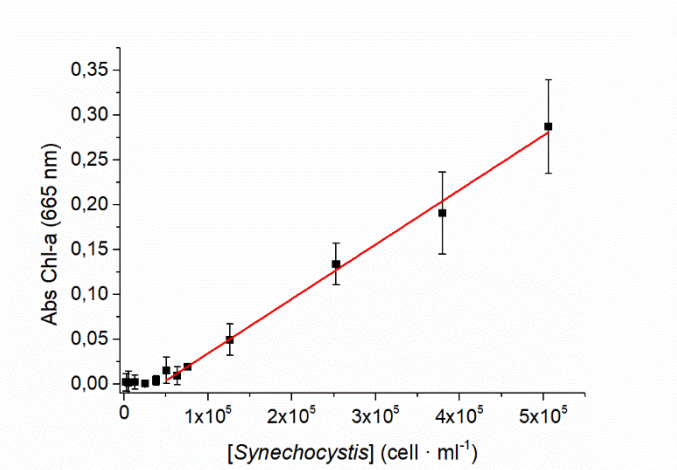

Figure 5.3. Calibration curve representing the absorbance of Chl-a at 665nm measured in samples with different cyanobacteria concentrations. The linear regression was calculated for the linear range (y = -0.026 + 6,07x10^{-7}x, R^2=0.99) and depicted in red.

5.3.3 On-chip pre-concentration and in situ determination of *Synechocystis* in water samples

The performance of the integrated platform was validated using water samples containing between 10^1 to 10^4 *Synechocystis* concentrations in cells·mL^{-1}. These samples were filtered and analyzed *in situ* with the optical components integrated into the system. With this aim, after sample filtration, the Luer-lock was replaced by a second PMMA piece with the LED and the detector so the fluorescence measurement could be done inside the system.

Figure 5.4 shows the emission spectra obtained with the system for cyanobacterial samples. These spectra presented two peaks at 665 nm and 680 nm, which were attributed to PhC and Chl-a [33,38], respectively. Both peaks were clearly appreciated at high bacterial concentrations and provide good linear correlations with bacterial concentration. However, PhC peak presented higher intensities at low concentrations and was more specific to cyanobacteria than Chl-a, which was also present in the majority of the components of the phytoplankton biomass. *E.coli* suspensions at a concentration of 10^6 cell·mL^{-1} were used as the negative control, which showed no fluorescence signal in the conditions of analysis. For these reasons, the fluorescence value at 665 nm was used in the following experiments to establish a correlation with cyanobacteria concentration.

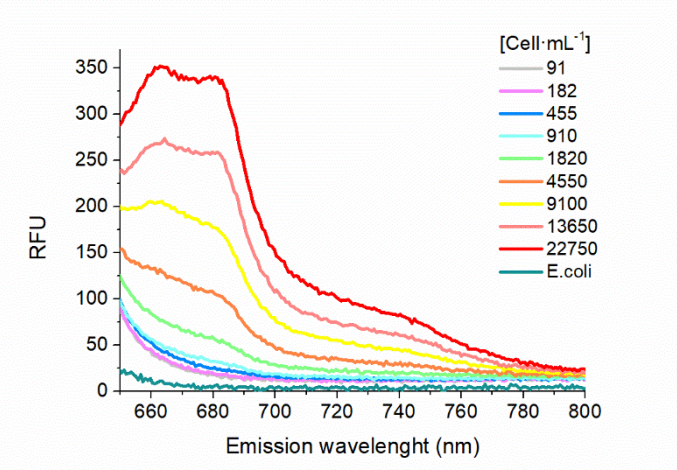

Figure 5.4. Emission spectra obtained after the excitation with a LED emitting at 590 nm for different Synechocystis concentrations (N=6). Arrows indicate the emission peaks corresponding to phycocyanins (665 nm) and chlorophyll-a (680 nm).

To improve fluorescence detection, the optical conditions of the measurement were analyzed and optimized. It is worth mentioning that the geometry of the fibers positioning angle in the holder was selected during the sensor's design to maximize the collection of emitted light while reducing interference from the background excitation light [48]. Apart from that, fluorescence measurements depended on the intensity of the emission source, integration time (IT), and photo-detector sensitivity. Since the light source intensity and the photo-detector sensitivity depended on the components and were already established, only the IT could be optimized. The IT is the length of time during which the detector collects photons before passing the accumulated voltage charge to a digital signal [53]. Longer ITs provide higher signal intensities up to a maximum accumulated voltage leading to detector saturation. However, noise also increases with the IT, which may affect the signal to noise ratio (SNR). The SNR is given by [54,55]:

$$\text{SNR} = \frac{Signal\ average}{Standad\ deviation} \qquad \text{Equation 5.5.}$$

Short IT of 15, 30 and 45 ms were evaluated for the fluorescence analysis of cyanobacteria. Results are represented in Figure 5.5A, with a magnification of the linear region for the low concentrations in Figure 5.5B.

In all cases, a similar linear range was obtained with a linear correlation between fluorescence magnitude and *Synechocystis* concentration until saturation around $6x10^4$ cell·mL^{-1}. The main differences were in the magnitude of the fluorescence intensity for each bacterial sample, which increased with the increasing IT. This increase, however, did not improve the analytical performance of the system, as shown in Table 5.1, where the limit of detection, quantification and the SNR are determined and presented for each IT. As shown, there was a small improvement in both LoD and LoQ when increasing the IT from 15 to 30 ms, but both parameters increased when increasing the IT to 45 ms. Similarly, the SNR improved from 15 to 30 ms but dramatically decreased when increasing the IT to 45 ms (Table 5.1).

An IT of 30 ms was selected accordingly in subsequent fluorescence measurements. Detection limits of about 435 cell·mL^{-1} with an R^2 = 0.99 could be reached. This was slightly higher to that obtained with a lab spectrometer (185 cell·mL^{-1}), but the instrumentation used is much simpler, lighter, portable and cost-effective than the latter.

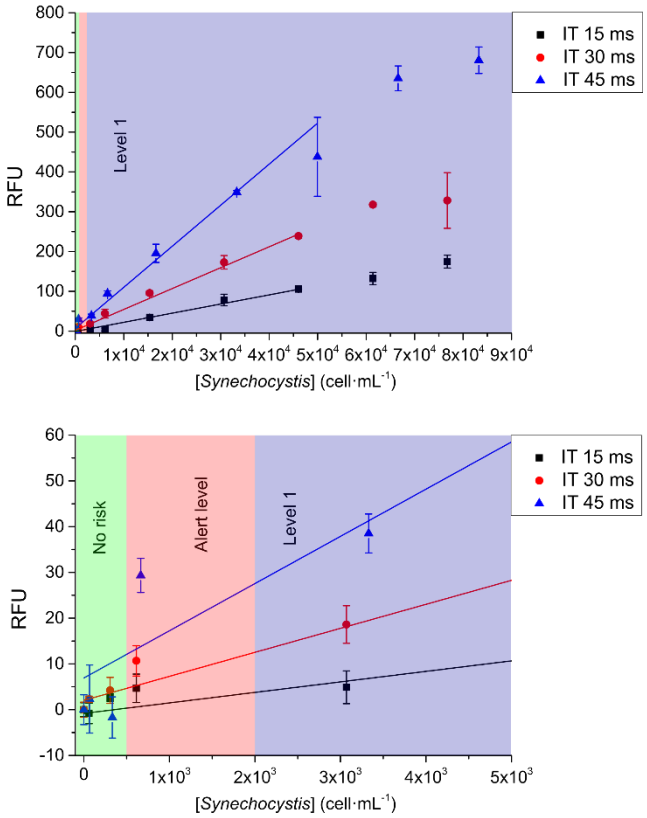

Figure 5.5. A) Calibration curve representing the relative fluorescence units at 665 nm obtained by different cyanobacteria concentrations using different ITs (15, 30, and 45 ms). Regression lines are depicted for the linear range of the curve. B) Zoom of the lowest concentrations of the calibrations curves. Error bars represent the standard deviation (n=3).

Table 5.1. Limits of detection (LoD) and quantification (LoQ) calculated for the different methods for cyanobacteria detection methods studied.

Detection method		Limits of the regression line		
		LoD (cell·mL^{-1})	LoQ (cell·mL^{-1})	
Chlorophyll extraction		1.3 x 10^4	4.8 x 10^4	
Microfiltration + Fluorescence in plate reader		185	616	SNR
Integrated device	IT 15 s	625	2078	9 ± 2
	IT 30 s	435	1455	16 ± 4
	IT 45 s	1292	4305	9 ± 3

5.3.4 Application of the photonic system in the analysis of aquaculture water samples

The integrated photonic system was finally applied to the detection of cyanobacteria in water samples from an aquaponics system. Water samples from three locations in the aquaponic system, i.e. the ID, the OF and the FS (Figure 5.6), where obtained and analyzed with the integrated photonic system and by a reference laboratory, by comparison.

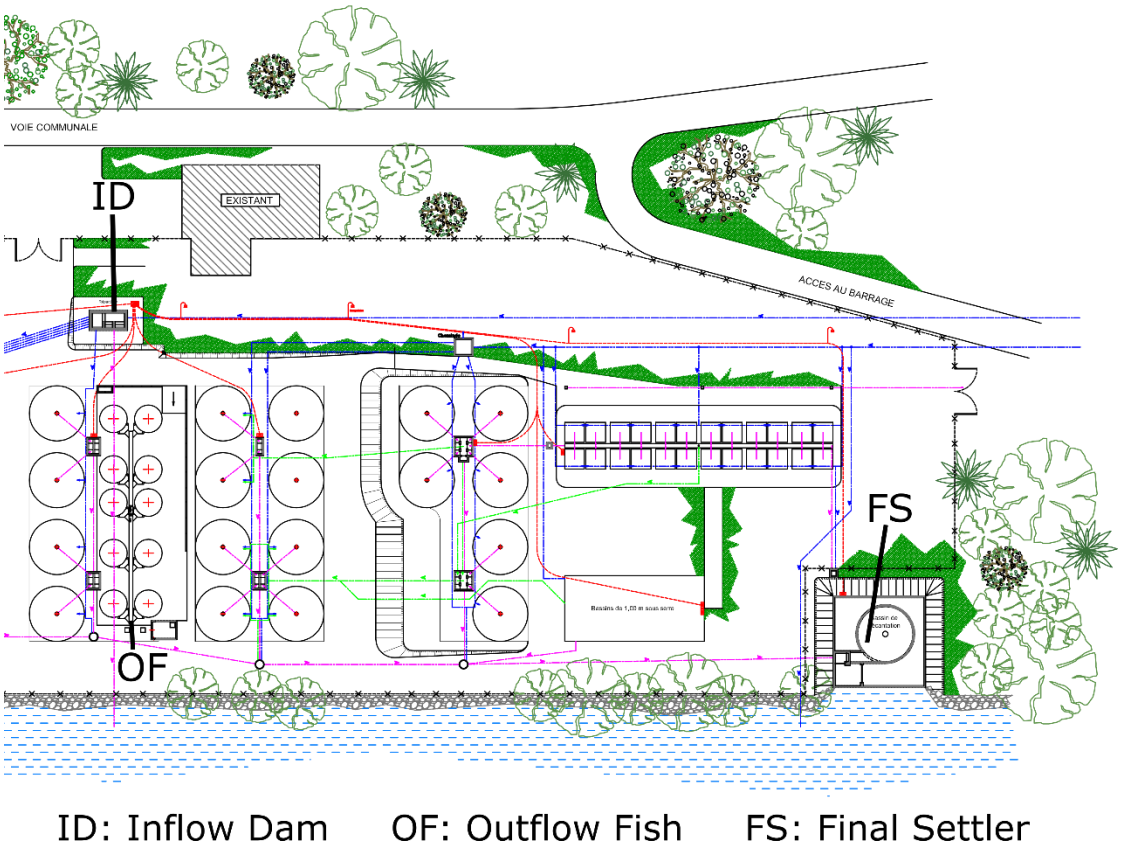

Figure 5.6. Schematic representation of the aquaculture compartment from the aquaponics system of PEIMA.

After sample concentration and fluorescence detection, the fluorescence values obtained for the OF, ID and FS was 1.6 RFU, 12.8 RFU and 48.83 RFU respectively, which corresponded to cyanobacterial concentrations below < 500 cell mL^{-1} in the case of OF, 2325 cell mL^{-1} for the ID and 9244 cell mL^{-1} for the FS. These values were in agreement with those provided by the reference laboratory, i.e. 11 cell mL^{-1} for OF, 3084 cell mL^{-1} for ID and 7508 cell mL^{-1} for FS, with small differences attributed to the transport, storage, ageing and manipulation of the samples which may be

minimized through in situ analysis with the current integrated photonic system. According to the results, OF water sample, obtained after filtration, did not represent a risk and could be recirculated along the aquaponics system; ID water, obtained directly from the lake, present cyanobacterial concentrations in the initial stage of the Level 1 and should be controlled since may become a risk for the fishes and may change their organoleptic properties; finally, FS water resulting from the convergence of all water in the aquaponics system presented high cyanobacterial concentrations in the Alert Level 1 and should not be recirculated in the aquaponics system since may be a risk for public health.

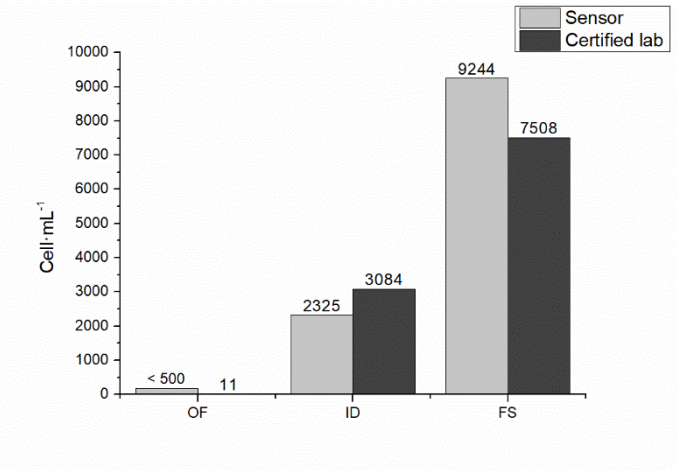

Figure 5.7. Cyanobacterial concentrations measured by the developed sensor (bight grey) and the certified lab (dark grey).

Thus, even with some discrepancies, the integrated photonic system provided results comparable to those obtained with standard methodologies but in a shorter time span thus enabling to prevent the devasting consequences, either economic or sanitary, of cyanobacterial blooms.

Besides conventional methods requiring tedious protocols based on benchtop equipment and, therefore, impossible to operate outside of the laboratory, PC fluorescence-based sensors such as the submersible field probes have already been developed. However, although submersible probes able to operate *in-situ* and give real-time results are commercially available (e.g., YSI 6600 and TriOS), these show lack of sensitivity below 10^3 cell·mL^{-1}, where the initial stage of the bloom already takes place. Moreover, similar approaches to ours describing compact detection

devices using *in vivo* fluorescence measurements can be found in the literature. Asai et al. (2000) [42], developed a system with a LED emitting at 620 nm and measuring at 645 nm capable of detecting cyanobacteria concentrations in the range of 10^3 – 10^6 cell·mL^{-1} in 25 minutes. The low LoD reached was due to pre-treatment with an ultrasonic device that allowed to disrupt the cyanobacterial cells and to extract the photosynthetic pigments, thus increasing the fluorescence response. Nevertheless, the implementation of a sonicator increments the size and cost of the device, hindering its portability and in situ application [41,42].

In another study, Shin et al. [29] proposed a 3 LED-based fluorescent sensor to stimulate the Chl-a and Chl-b in eukaryotic algae and PhC in cyanobacteria. Even though the sensor demonstrated the ability to detect different phytoplankton species, they only achieved an LoD for cyanobacteria of 4 mg·L^{-1}, corresponding to concentrations above 10^6 cell ·mL $^{-1}$. Therefore, the system could be valid only for the identification of species in established bloom assemblages and not as a method for early prevention of cyanobacterial blooms.

Instead, our system reaches a detection limit of 435 cell·mL^{-1} by integrating two steps, namely pre-concentration and fluorescence measurement, in a miniaturized, portable and robust system enabling in situ analysis. Besides, the time required to carry out the whole process is about 2 min. When the analyzed samples corresponded to real samples from aquaculture systems, this measurement time could be increased depending on the number of particles (e.g., fish food remains) present in the samples. Even so, it did not exceed 10 minutes, which is more than an adequate time to have an early warning of the presence of bacteria to prevent cyanobacterial blooms.

5.4 CONCLUSION

To address the need for early warning of cyanobacterial blooms, this work presents an integrated photonic system combining bacterial pre-concentration by filtration in NC filters and in situ fluorescence detection of cyanobacteria through specific pigment PhC. The system is compact, portable and robust, and shows an excellent correlation between the measured fluorescence and cyanobacteria concentration in

a range between 10^2 – 10^4 cell·mL^{-1}. With a detection limit of 435 cells·mL^{-1}, a value within the pre-bloom Alert Level stages established by the World Health Organization; the system is sensitive enough to identify cyanobacterial blooms in their early stages, preventing their fast and hazardous proliferation. Moreover, the whole process takes less than 10 minutes with a fully automated signal acquisition via Raspberry pi, which overcomes the long assay times of conventional methods, the need for qualified personnel and the use of sophisticated and expensive laboratory equipment.

Thanks to its simplicity, portability and quick response, we envision the current system as a powerful alternative for early warning of cyanobacterial bloom, allowing to perform analysis at different points of installations such as water treatment plants or aquaculture systems, where cyanobacteria bloom is a big problem, or even in natural water environments as lakes, predicting blooms quickly and efficiently, without the need of qualified personnel.

REFERENCES

[1] L. Brient, M. Lengronne, E. Bertrand, D. Rolland, A. Sipel, D. Steinmann, I. Baudin, M. Legeas, B. Le Rouzic, M. Bormans, A phycocyanin probe as a tool for monitoring cyanobacteria in freshwater bodies, J. Environ. Monit. 10 (2008) 248–255. https://doi.org/10.1039/B714238B.

[2] H. Chakdar, S. Saha, S. Pabbi, Chromatographic and spectroscopic characterization of phycocyanin and its subunits purified from Anabaena variabilis CCC421, Appl. Biochem. Microbiol. 50 (2014) 62–68. https://doi.org/10.1134/S0003683813060057.

[3] Environmental Protection Agency, Cyanobacteria and Cyanotoxins: Information for Drinking Water Systems, United States Environ. Prot. Agency. (2014) 1–9. https://www.epa.gov/sites/production/files/2014-08/documents/cyanobacteria_factsheet.pdf (accessed March 19, 2018).

[4] Y. Blanco, A. Quesada, I. Gallardo-Carreño, J. Aguirre, V. Parro, CYANOCHIP: An antibody microarray for high-taxonomical-resolution cyanobacterial monitoring, Environ. Sci. Technol. 49 (2015) 1611–1620. https://doi.org/10.1021/es5051106.

[5] J. Gregor, B. Maršálek, A simple in vivo fluorescence method for the selective detection and quantification of freshwater cyanobacteria and eukaryotic algae, Acta Hydrochim. Hydrobiol. 33 (2005) 142–148. https://doi.org/10.1002/aheh.200400558.

[6] H.W. Paerl, R.S. Fulton, P.H. Moisander, J. Dyble, Harmful freshwater algal blooms, with an emphasis on cyanobacteria., ScientificWorldJournal. 1 (2001) 76–113. https://doi.org/10.1100/tsw.2001.16.

[7] H.W. Paerl, T.G. Otten, Harmful Cyanobacterial Blooms: Causes, Consequences, and Controls, Microb. Ecol. 65 (2013) 995–1010. https://doi.org/10.1007/s00248-012-0159-y.

[8] J. Ndiritu, R. Wanjau, J. Murungi, International Journal for Innovation Education and Research, Int. J. Innov. Educ. Res. 1 (2013) 95–108. http://ijier.net/ijier/article/view/973 (accessed April 3, 2018).

[9] A.D. Turner, M. Dhanji-Rapkova, A. O'Neill, L. Coates, A. Lewis, K. Lewis,

Analysis of microcystins in cyanobacterial blooms from freshwater bodies in England, Toxins (Basel). 10 (2018) 39. https://doi.org/10.3390/toxins10010039.

[10] R. Suneerat, Y. Wichien, T. Paveena, G. Monthon, Cyanobacterial Composition and Microcystin Accumulation in Catfish Pond, Chiang Mai J. Sci. 41 (2014) 27–38. http://epg.science.cmu.ac.th/ejournal/ (accessed January 24, 2018).

[11] A. Sinden, S.C. Sinang, Cyanobacteria in aquaculture systems: linking the occurrence, abundance and toxicity with rising temperatures, Int. J. Environ. Sci. Technol. 13 (2016) 2855–2862. https://doi.org/10.1007/s13762-016-1112-2.

[12] Z. Li, S. Zhang, T. Yu, Z. Dai, Q. Wei, Aptamer-Based Fluorescent Sensor Array for Multiplexed Detection of Cyanotoxins on a Smartphone, (2019). https://doi.org/10.1021/acs.analchem.9b00750.

[13] F. Choo, A. Zamyadi, K. Newton, G. Newcombe, L. Bowling, R. Stuetz, R.K. Henderson, Performance evaluation of in situ fluorometers for real-time cyanobacterial monitoring, H2Open J. 1 (2018) 26–46. https://doi.org/10.2166/h2oj.2018.009.

[14] B. K. Dwivedi, Potential Risk Assessment of Cyanotoxin for Water Resources, Int. J. Curr. Microbiol. Appl. Sci. 7 (2018) 3604–3616. https://doi.org/10.20546/ijcmas.2018.702.429.

[15] J.M. O'Neil, T.W. Davis, M.A. Burford, C.J. Gobler, The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change, Harmful Algae. 14 (2012) 313–334. https://doi.org/10.1016/j.hal.2011.10.027.

[16] W. Teng, Z. Wu, J. Fan, H. Chen, D. Feng, Y. Lv, J. Wang, A.M. Asiri, D. Zhao, Ordered mesoporous carbons and their corresponding column for highly efficient removal of microcystin-LR, Energy Environ. Sci. 6 (2013) 2765–2776. https://doi.org/10.1039/c3ee41775a.

[17] G.R. Shaw, D.P. Moore, C. Garnett, Eutrophication and Algal Blooms, Encycl. Life Support Syst. 2 (2003) 1–21. http://www.eolss.net/Sample-Chapters/C06/E6-13-04-04.pdf (accessed April 3, 2018).

[18] K.G. Sellner, G.J. Doucette, G.J. Kirkpatrick, Harmful algal blooms: Causes,

impacts and detection, J. Ind. Microbiol. Biotechnol. 30 (2003) 383–406. https://doi.org/10.1007/s10295-003-0074-9.

[19] J. Huisman, G.A. Codd, H.W. Paerl, B.W. Ibelings, J.M.H. Verspagen, P.M. Visser, Cyanobacterial blooms, Nat. Rev. Microbiol. 16 (2018) 471–483. https://doi.org/10.1038/s41579-018-0040-1.

[20] N. McQuaid, A. Zamyadi, M. Prévost, D.F. Bird, S. Dorner, Use of in vivophycocyanin fluorescence to monitor potential microcystin-producing cyanobacterial biovolume in a drinkingwater source, J. Environ. Monit. 13 (2011) 455–463. https://doi.org/10.1039/C0EM00163E.

[21] W.A. Wurtsbaugh, H.W. Paerl, W.K. Dodds, Nutrients, eutrophication and harmful algal blooms along the freshwater to marine continuum, Wiley Interdiscip. Rev. Water. 6 (2019) e1373. https://doi.org/10.1002/wat2.1373.

[22] N.-Y. Benayache, T. Nguyen-Quang, K. Hushchyna, K. McLellan, F.-Z. Afri-Mehennaoui, N. Bouaïcha, An Overview of Cyanobacteria Harmful Algal Bloom (CyanoHAB) Issues in Freshwater Ecosystems, in: Limnol. - Some New Asp. Inl. Water Ecol., IntechOpen, 2019. https://doi.org/10.5772/intechopen.84155.

[23] J. Liu, X. Zhou, H. Shi, An Optical Biosensor-Based Quantification of the Microcystin Synthetase A Gene: Early Warning of Toxic Cyanobacterial Blooming, Anal. Chem. 90 (2018) 2362–2368. https://doi.org/10.1021/acs.analchem.7b04933.

[24] J.M.E. Kasinak, B.M. Holt, M.F. Chislock, A.E. Wilson, Benchtop fluorometry of phycocyanin as a rapid approach for estimating cyanobacterial biovolume, J. Plankton Res. 37 (2014) 248–257. https://doi.org/10.1093/plankt/fbu096.

[25] J. Chorus, Ingrid & Bartram, Toxic cyanobacteria in water. A guide to their public health consequences, monitoring, and management / edited by Ingrid Chorus and Jamie Bertram., World Heal. Organ. (1999). http://www.earthprint.com (accessed August 26, 2019).

[26] C. Jin, M.M.F. Mesquita, J.L. Deglint, M.B. Emelko, A. Wong, Quantification of cyanobacterial cells via a novel imaging-driven technique with an integrated fluorescence signature, Sci. Rep. 8 (2018) 9055. https://doi.org/10.1038/s41598-018-27406-0.

[27] K. Izydorczyk, M. Tarczynska, T. Jurczak, J. Mrowczynski, M. Zalewski, Measurement of phycocyanin fluorescenceas an online early warning system for cyanobacteria in reservoir intake water, Environ. Toxicol. 20 (2005) 425–430. https://doi.org/10.1002/tox.20128.

[28] K. Izydorczyk, C. Carpentier, J. Mrówczyński, A. Wagenvoort, T. Jurczak, M. Tarczyńska, Establishment of an Alert Level Framework for cyanobacteria in drinking water resources by using the Algae Online Analyser for monitoring cyanobacterial chlorophyll a, Water Res. 43 (2009) 989–996. https://doi.org/10.1016/j.watres.2008.11.048.

[29] Y.-H. Shin, J.Z. Barnett, M.T. Gutierrez-Wing, K.A. Rusch, J.-W. Choi, A hand-held fluorescent sensor platform for selectively estimating green algae and cyanobacteria biomass, Sensors Actuators B Chem. 262 (2018) 938–946. https://doi.org/10.1016/J.SNB.2018.02.045.

[30] E. Bertone, A. Chuang, M.A. Burford, D.P. Hamilton, In-situ fluorescence monitoring of cyanobacteria: Laboratory-based quantification of species-specific measurement accuracy, Harmful Algae. 87 (2019) 101625. https://doi.org/10.1016/j.hal.2019.101625.

[31] P. Yeh, N. Yeh, C.H. Lee, T.J. Ding, Applications of LEDs in optical sensors and chemical sensing device for detection of biochemicals, heavy metals, and environmental nutrients, Renew. Sustain. Energy Rev. 75 (2017) 461–468. https://doi.org/10.1016/j.rser.2016.11.011.

[32] M. Beutler, K.H. Wiltshire, M. Arp, J. Kruse, C. Reineke, C. Moldaenke, U.P. Hansen, A reduced model of the fluorescence from the cyanobacterial photosynthetic apparatus designed for the in situ detection of cyanobacteria, Biochim. Biophys. Acta - Bioenerg. 1604 (2003) 33–46. https://doi.org/10.1016/S0005-2728(03)00022-7.

[33] A. Zamyadi, F. Choo, G. Newcombe, R. Stuetz, R.K. Henderson, A review of monitoring technologies for real-time management of cyanobacteria: Recent advances and future direction, TrAC - Trends Anal. Chem. 85 (2016) 83–96. https://doi.org/10.1016/j.trac.2016.06.023.

[34] L. Zeng, D. Li, Development of in situ sensors for chlorophyll concentration measurement, J. Sensors. 2015 (2015) 1–16. https://doi.org/10.1155/2015/903509.

[35] H. Schubert, U. Schiewer, E. Tschirner, Fluorescence characteristics of cyanobacteria (blue-green algae), J. Plankton Res. 11 (1989) 353–359. https://doi.org/10.1093/plankt/11.2.353.

[36] G. Persichetti, E. Viaggiu, G. Testa, R. Congestri, R. Bernini, Spectral discrimination of planktonic cyanobacteria and microalgae based on deep UV fluorescence, Sensors Actuators, B Chem. 284 (2019) 228–235. https://doi.org/10.1016/j.snb.2018.12.111.

[37] T.- Alam, Extraction of Natural Colors from Marine Algae, J. Agric. Mar. Sci. [JAMS]. 23 (2019) 81. https://doi.org/10.24200/jams.vol23iss1pp81-91.

[38] J. Gregor, B. Maršálek, H. Šípková, Detection and estimation of potentially toxic cyanobacteria in raw water at the drinking water treatment plant by in vivo fluorescence method, Water Res. 41 (2007) 228–234. https://doi.org/10.1016/j.watres.2006.08.011.

[39] S.H. Lee, V. Mariappan, D.C. Won, M. Ann, S. Yang, Design of Optical Biological Sensor for Phycocyanin Parameters Measurement using Fluorescence Technique, Int. J. Adv. Smart Converg. 5 (2016) 73–79. https://doi.org/10.7236/IJASC.2016.5.2.73.

[40] E. Bertone, M.A. Burford, D.P. Hamilton, Fluorescence probes for real-time remote cyanobacteria monitoring: A review of challenges and opportunities, Water Res. 141 (2018) 152–162. https://doi.org/10.1016/j.watres.2018.05.001.

[41] C. Bastien, R. Cardin, É. Veilleux, C. Deblois, A. Warren, I. Laurion, Performance evaluation of phycocyanin probes for the monitoring of cyanobacteria, J. Environ. Monit. 13 (2011) 110–118. https://doi.org/10.1039/c0em00366b.

[42] R. Asai, S. McNiven, K. Ikebukuro, I. Karube, Y. Horiguchi, S. Uchiyama, A. Yoshida, Y. Masuda, Development of a Fluorometric Sensor for the Measurement of Phycobilin Pigment and Application to Freshwater Phytoplankton, F. Anal. Chem. Technol. 4 (2000) 53–61. https://doi.org/10.1002/(SICI)1520-6521(2000)4:1<53::AID-FACT6>3.0.CO;2-C.

[43] Adel M. Mahasneh, S.M. Bdour, Microbiology Laboratory Manual, AMMAN, 2006.

[44] A. Bennett, L. Bogobad, Complementary chromatic adaptation in a filamentous blue-green alga, J. Cell Biol. 58 (1973) 419–435. https://doi.org/10.1083/jcb.58.2.419.

[45] J.J. Ezenarro, N. Uria, Ó. Castillo-Fernández, N. Párraga, M. Sabrià, F.X. Muñoz Pascual, Development of an integrated method of concentration and immunodetection of bacteria, Anal. Bioanal. Chem. 410 (2018) 105–113. https://doi.org/10.1007/s00216-017-0695-x.

[46] T.N. Ackermann, P. Giménez-Gómez, X. Muñoz-Berbel, A. Llobera, Plug and measure-a chip-to-world interface for photonic lab-on-a-chip applications, Lab Chip. 16 (2016) 3220–3226. https://doi.org/10.1039/c6lc00462h.

[47] Files · RPi · Tobias Nils Ackermann / PythonLab · GitLab, (n.d.). https://gitlab.com/TobiasNils/PythonLab/tree/RPi (accessed September 4, 2019).

[48] J. Janata, Principles of Chemical Sensors, Springer US, 2009. https://doi.org/10.1007/b136378.

[49] A. Zamyadi, N. McQuaid, M. Prévost, S. Dorner, Monitoring of potentially toxic cyanobacteria using an online multi-probe in drinking water sources, J. Environ. Monit. 14 (2012) 579–588. https://doi.org/10.1039/c1em10819k.

[50] N. Párraga-Niño, S. Quero, A. Ventós-Alfonso, N. Uria, O. Castillo-Fernandez, J.J. Ezenarro, F.X. Muñoz, M. Garcia-Nuñez, M. Sabrià, New system for the detection of Legionella pneumophila in water samples, Talanta. 189 (2018) 324–331. https://doi.org/10.1016/j.talanta.2018.07.013.

[51] N. Krishnaveni, A. Shenbaga Devi, P. Santhanam, A Method of Analysis of Pigments in Phytoplankton, in: Basic Appl. Phytoplankt. Biol., Springer Singapore, Singapore, 2019: pp. 221–227. https://doi.org/10.1007/978-981-10-7938-2_11.

[52] A. Lall, S.A. John, Cyanobacterial pigments analysis extracted from different sources of District Allahabad, ~ 1413 ~ J. Pharmacogn. Phytochem. 6 (2017) 1413–1415. https://pdfs.semanticscholar.org/c8d6/72eeabd6cc2a5ff9ccef8e4fb1fab5667cf7.pdf (accessed July 15, 2019).

[53] A Glossary of Spectroscopic Terms - Ocean Optics, (n.d.).

https://oceanoptics.com/glossary/ (accessed August 21, 2019).

[54] D.J. Schroeder, Astronomical Optics, Second Edition, Academic Press, 1999. http://www.amazon.com/Astronomical-Optics-Second-Edition-Schroeder/dp/0126298106 (accessed November 6, 2019).

[55] J.T. Bushberg, The essential physics of medical imaging, Lippincott Williams & Wilkins, 2002.

6. PORTABLE PHOTONIC PROTOTYPE FOR THE CONCENTRATION AND DETECTION OF BACTERIA

ABSTRACT

According to current regulations, microbial quality standards in water and food involve the detection of low but clinically significant bacterial concentrations. To reach this low detection limits, four strategies are currently used, namely bacterial enrichment, signal amplification, preconcentration or the use of single-cell analysis techniques. Among them, preconcentration is now a first choice in the development of in situ analysis systems for its simplicity, low-cost and possibility of integration and miniaturization. However, a fully integrated system enabling sample preconcentration and in situ detection out of the laboratory has not been developed yet. Here, we present a portable, low-cost and user-friendly photonic device integrating fluidic and optical components for the rapid processing of large sample volumes and in situ bacterial detection, here applied to the waterborne pathogen cyanobacteria. Fluidically, the system integrates a high-performance filtration system that allows the processing of 1 L in about 40 min widely surpassing the performance of any other system published up to now. Additionally, the system incorporates two optical fibers at 45º for in situ optical analysis of the retained microorganisms. Spectroscopic analysis of the sample allows for bacterial identification through specific autofluorescence bands, in this case, associated to chlorophyll and phycocyanin pigments. The quantitative analysis of the phycocyanin band is here employed to quantify low cyanobacteria concentration between 10^2 to 10^4 cell·mL^{-1} (R^2=0.99) for early detection of cyanobacterial blooms. The device is sensitive and fast, showing a limit detection below 480 cells·mL^{-1} after 10 minutes of preconcentration. Combining specific spectroscopic analysis, e.g. Mie-backscattering and autofluorescence, the device may be used for the identification of other pathogens in water samples, e.g. *Escherichia coli*. Hence, we envision the current prototype as a powerful tool for early warning of bacterial contamination.

6.1 INTRODUCTION

The presence of bacteria in water or food, even at low concentrations, may have catastrophic consequences including disease, hospitalization or death. For this reason, early detection and identification of pathogens are essential to guarantee the quality and safety of water and food, and to prevent infections from pathogenic bacteria [1,2]. Regulatory bodies and agencies are well aware of this problem and current regulations limit the presence of bacteria below 1 colony forming unit (CFU) in 25 mL (or 25 mg) in food and to 1 CFU per 100 mL in water [3,4]. To reach this low concentrations, two main strategies are now employed in the laboratory settings, i.e. enrichment and signal amplification. In the first case, bacteria cultured in specific media and conditions producing colonies that allow their identification and quantification. Although still the reference method, cell culturing is now being replaced by faster technologies (it may require from days to weeks), more general (it is limited to viable and cultural organisms [5]) and less subjective (it depends a lot on the experience of the user). Signal amplification techniques, and among them the polymerase chain reaction (PCR), is positioning as the best alternative to traditional methods. In this case, microorganisms are identified by the recognition of specific oligonucleotide sequences, which amplified in a chain reaction by the polymerase. Quantitative results are provided after less than 4 hours. Although some portable systems exist, this is mostly a laboratory technique that requires dedicated instrumentation and expensive reagents (e.g., primers) for a selective detection, as well as trained personnel [6–8]. Moreover, PCR is restricted to small sample volumes between 10 and 50 μL, while the regulation clearly establishes the analysis of large volumes: 25 mL (or g) [3] for food and up to 100 mL [9–11] in the case of water respectively.

A number of strategies have been developed in the last decades aiming to detect these low bacterial concentrations, such as highly sensitive immunoassays, biochips, biosensors or even single-cell analysis techniques, e.g. SERS. However, they work at the microscale (< 1 mL) and a previous reduction of the sample volume is needed before analysis [12]. Many preconcentration methods have been developed with the double function of reducing sample volume and concentrating bacteria for sensitive detection [13]. Due to the limited concentration capacity of immunomagnetic separation (IMS) systems [14–17] and the incapacity to process large sample volumes of those based on centrifugation [21,22], membrane filtration is considered a first

choice in the development of portable systems for in situ detection of bacteria [23,24]. In previous works, we have developed a high-performance microfiltration platform enabling the processing of high sample volumes in short times. The selective on-filter detection with specific antibodies was used to selectively detect and quantify *Escherichia coli* [25] and *Legionella pneumophila* [26] through electrochemical/optical transduction out of the system. As a step forward, optical fibers were integrated in the filtration structure for direct bacterial detection on the system but still requiring the plugging/unplugging of the fluidic/optical elements, i.e., light source and spectrometer, which compromised the system portability as well its performance and reliability.

In this work, a fully-integrated, portable, miniaturized and high-performance prototype incorporating all the necessary fluidic and optic components is presented. This platform has the advantage of enabling simultaneous sample processing, i.e., bacterial retention in microfiltration membranes and spectroscopic on-membrane analysis without the need of manipulation of the structure, thus providing real-time information of the filtration process. Spectroscopic analysis of the membrane allows in situ and real time identification and quantification of the bacteria retained on the filter through species-specific autofluorescence patterns. As a proof-of-concept, it has been applied to the detection of low cyanobacteria concentration (below 500 cells·mL^{-1}) as an early warning system of cyanobacterial blooms [34] just before that they compromise water quality due to toxin production.

6.2 MATERIALS AND METHODS

6.2.1 Cyanobacteria culture, sample preparation and quantification

Synechocystis sp. PCC 6803 was used as model cyanobacteria. Cyanobacteria were cultivated at 18°C in BG-11 medium (Sigma-Aldrich) containing trace metal mix A5 (Sigma-Aldrich) under dark:light cycles (12:12 h) at 6000 lx. Before experiments, several dilutions were prepared in the range from 10^1 to 10^5 cells mL^{-1} in distilled water. Each dilution was prepared in triplicate.

The concentration of the initial culture of *Synechocystis* sp. was determined by fluorescence microscopy in a Zeiss Imager M2 microscope by the Breeds counting equation (Equation 6.1) [39].

$$Cell \cdot mL^{-1} = \frac{n°cells \times (analised\ area/microscope\ field)}{volume \times dilution} \qquad \text{Equation 6.1.}$$

Where $n°\ cells$ is the average of cell numbers at ten different fields of the holder, *analyzed area* is the area where the sample is spread over, *microscope field* is the area observed under the microscope (40X = 0.08 mm^2), *volume* is the sample volume analyzed at the microscope and *dilution* indicates the dilution of the initial culture.

6.2.4 Portable prototype for cyanobacteria detection

6.2.4.1 Prototype design and fabrication

A 3D model of the preconcentration *holder* was designed based on the photonic device presented in chapter 5 using VectorWorks 2018 (Techlimits, ES) and mechanized by fast-prototyping techniques. Concerning the fluidic part, a disk-shaped piece of 9 mm of thickness and 50 mm of diameter containing a 1.2 mL reservoir was fabricated with a micromilling machine (Roland MDX-40, Roland Digital Group Iberia, Spain). The piece had a 2-mm of diameter central hole working as microfluidic inlet and an O-ring integrated at the top piece was used to avoid the fluid leakage. Regarding the bottom structure forming the fluidic part, it was defined in the top of the box containing the electronic components and the reservoir used to collect the fluidic waste after the filtration. Finally, both structures were fixed with four screws.

The remaining optical elements were implemented in a second polymeric structure, formed with two extra PMMA pieces fabricated by using a CO2-laser system controlled by a PC (Epilog Mini 24, Epilog Laser, USA). One permanently attached with epoxy adhesive at the top of the structure to seal and position the optical fiber (2-mm thick) and the other to position the light-emitting diode and the detector plugging (5-mm thick).

Finally, a box containing the bottom fluidic structure described above and the electronic part was fabricated by using a CO_2-laser system. The box was formed by

seven sheets of 7-mm thick of PMMA, with a total size of 180 mm x 75 mm x 106 mm (length x width x height).

6.2.4.2 Integrated photonic prototype operation for *Synechocystis* quantification

A nitrocellulose (NC) membrane (Amersham™ Protran™, GE Healthcare) was placed inside the *holder* for bacterial preconcentration. A total volume of 15 mL was filtered through the *holder* by a syringe. After filtration, fluorescence measurements were carried out. In the same way, 15 mL of water samples containing *E.coli* at a concentration of 10^6 cell·mL^{-1} were used as negative control.

Fluorescence measurements of the photosynthetic pigment phycocyanin were taken from the central filter area (~ 1 mm^2) to estimate the *Synechocystis* concentration present in the water sample. Emission spectra for each sample were obtained after subtraction of the dark spectrum obtained when the LED was switched OFF. Therefore, the recording of one spectrum (N = 1) required a measurement cycle comprised of a period of signal acquisition without excitation (dark spectrum acquisition) followed by the second period of identical duration (5 ms) to register the emission of the sample after excitation. Each dark and excitation measurements were recorded 10 times, and an average spectrum was obtained. The dark measurements were used to normalize the excited measurements before being represented.

After the selective excitation of the cyanobacterial pigment phycocyanin at 590 nm, fluorescence emission was measured at 660 nm. All measurements were carried out by triplicate, and the averages and standard deviations were calculated.

Finally, a calibration curve was obtained by representing the intensity of the fluorescence peak emission at 660 nm in Relative Fluorescence Units (RFU) versus cyanobacterial concentration (*Synechocystis* cells mL^{-1}). Thus, the values in the linear range were used to determine the regression line, the limit of detection (LoD) and the limit of quantification (LoQ) using the following equations:

$$LoD = \frac{3 \times SDa}{b} \qquad \text{Equation 3.}$$

$$LoQ = \frac{10 \times SDa}{b} \qquad \text{Equation 4.}$$

Where SDa is the standard error of the response and b is the slope.

6.3 RESULTS AND DISCUSSION

6.3.1 Portable prototype for in situ cyanobacteria bloom prevention

The concentration and detection platform

To obtain a user-friendly and portable cyanobacteria detection prototype, a new device has been designed based on the photonic device fabricated in Chapter 5, but this time incorporating all fluidic/optical components in a single fully integrated, miniaturized and portable photonic system enabling simultaneous sample processing and spectroscopic on-membrane analysis.

The new holder consists of two parts of 50 mm of diameter assembled by screws and sealed by a rubber O-ring (20 mm of inner diameter and 3 mm in width) to ensure water tightness (Figure 6.1). The upper part (9 mm of width), contained (A) a 2 mm diameter central hole working as fluidic inlet, (B) a cavity containing a 1,2 mL reservoir with (C) two holes of 1 mm at 45° angle relative to the z-axis for the incorporation of the flexible optical fibers (Keyence, ToughFlex (613 core) fibers); and (D) a second cavity to implement the rubber O-ring used to avoid the water leakage. The lower part was defined in the top of the box containing the electronic components and contained (E) a planar socket with evacuation channels to place the nitrocellulose (NC) membrane used for cell retention and preconcentration and (F) a fluidic outlet.

The design also included two PMMA pieces divided in two parts for the integration and positioning of the optical components required for the fluorescence measurement in situ. The first one (G) was attached permanently to the top layer of the holder with epoxy adhesive. The second PMMA piece (H) contained a cavity for the suitable where (I) a LUXEON Rebel “amber” LumiLED light-emitting diode (LED) with an emission peak at 590 nm was permanently positioned, and (J) a plug connection [40] for a (K) SMA-connected optical fiber (QP600-1-XSR, Ocean Optics, Largo, FL, USA) of 600 μm/1000 μm core/cladding diameter, which was connected to an external spectrometer (C12985-10, Hamamatsu Photonics) used as the optical detector. The simple assembly between both PMMA pieces ensured the alignment of the optical components (LED and detector) with the flexible optical fibers.

Optical components for fluorescence measurements were controlled and synchronized through a custom-made software based on Python [41].

Finally, a PMMA box (L) has been fabricated to integrate the concentration and detection holder and (M) spectrometer together with a custom printed circuit board (PCB) incorporating a (N) Raspberry Pi microprocessor used for control and synchronize all optical components. Two closed compartments hosted the electronic components in order to avoid fluidic contact, and a third compartment was used to host the (O) 50 mL reservoir employed as waste collector. The different walls of the box can be easily disassembled by using a screwdriver to empty the waste collector or adjust some electronic components.

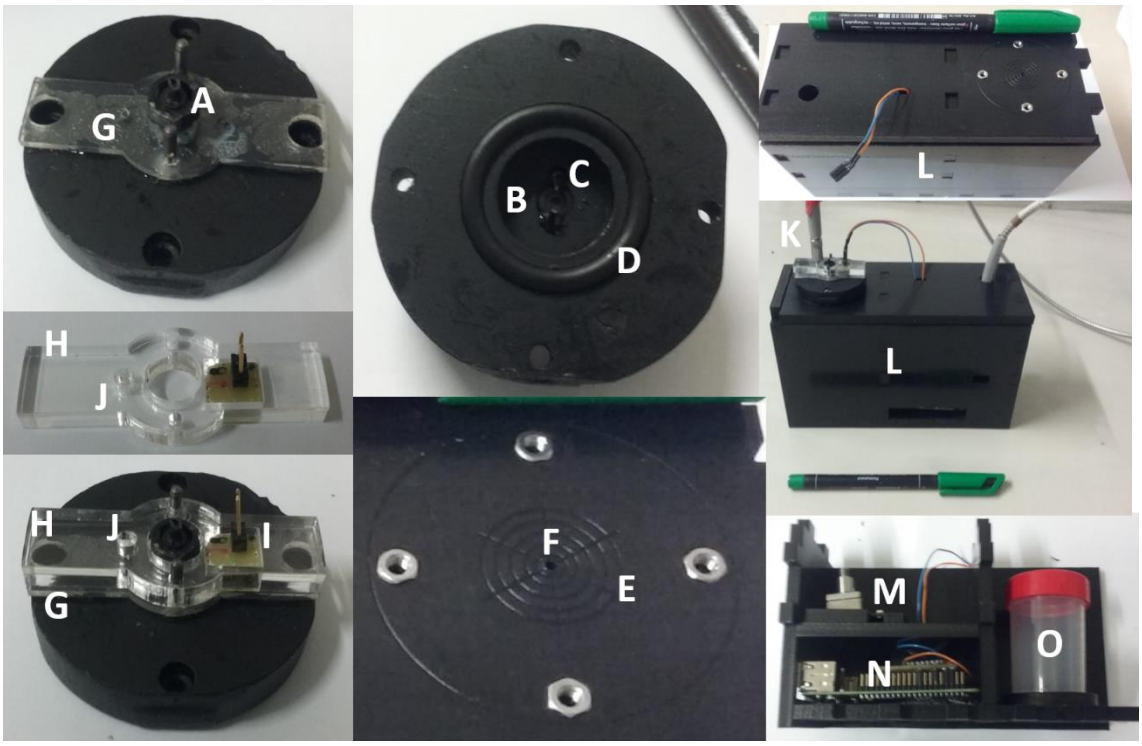

Figure 6.1. Photographs of the different parts comprising the portable photonic device.

Validation of the miniaturized spectrometer

To achieve a compact photonic device and gain in portability, a miniaturized and cost-effective mini-spectrometer provided by Hamamatsu was selected to carry out fluorescence analysis. The performance of the mini-spectrometer was compared to the high-performance benchtop spectrometer QEPro from Ocean Optics (Ocean Optics, Largo, FL, USA). To this end, the fluorescence of different cyanobacteria concentrations previously retained in the filter membranes of the prototype was

determined with both spectrometers and compared. A good correlation (R^2=0.98) was obtained (Figure 6.2) between them, which validated the use of the mini-spectrometer even when much smaller (50 x 40 x 29.3 mm vs 182 x 110 x 47 mm), lighter (45 vs 1500 g) and cheaper (200 € Hamamatsu vs 9000€ Ocean optics).

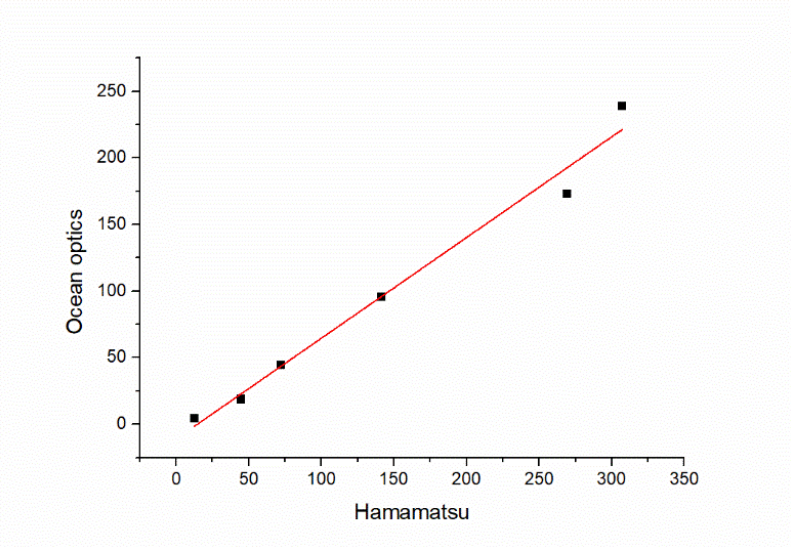

Figure 6.2. Correlation of the relative fluorescence units obtained for different cyanobacterial concentrations by the Hamamatsu spectrometer vs the Ocean Optics.

6.3.2 Prototype testing

In order to validate the performance of the prototype, it was tested for cyanobacteria detection using water samples containing between 10^1 to 10^4 *Synechocystis* cells·mL^{-1}. After cyanobacteria retention, the emission spectra obtained was recorded. Each spectrum was normalized by the corresponding dark measurement. In the same way, 15 mL of water samples containing *E.coli* at a concentration of 10^6 cell·mL^{-1} were used as the negative control, which showed no fluorescence signal in the conditions of analysis.

The fluorescence emission value at 660 nm, corresponding to PhC was used to establish a correlation with cyanobacteria concentration (Figure 6.3). It could be appreciated that as higher was the *Synechocystis* concentration present in the sample, higher was the obtained fluorescence magnitude. Hence, a calibration curve maintaining a good linear correlation with an R^2 value of 0.99 and an LoD of 480 cell·mL^{-1} was obtained. The prototype is able to support flow rates as high as 30

mL·min^{-1} offering the possibility to process large sample volumes at a short time. Therefore, the limit of detection could be even improved, being extremely sensitive and reaching detection limits even lower than 1 CFU·mL^{-1}.

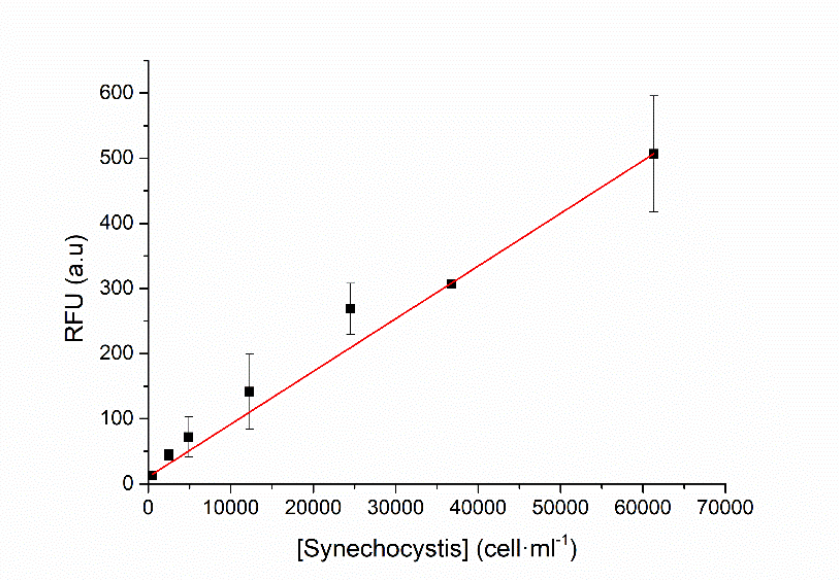

Figure 6.3. Calibration curve representing the relative fluorescence units obtained by different cyanobacteria concentrations. Error bars represent the standard deviation.

Many fluorimeters are available in the market, offering the possibility to detect different pigments and physical parameters of water (pH, temperature, etc.). However, many of them are bulky and present high prices [42], in addition to present hard reading results given as µg·L^{-1} of pigment what makes difficult the correlation to the cyanobacteria cell concentration of the analyzed waters.

In contrast, our device is able to correlate the fluorescence magnitude to cyanobacterial concentration, offering easy to interpret results. The prototype has an LoD of 480 cell·mL^{-1}, in the same order of magnitude of some commercial probes such as YSI 6600 V2 and MS5 but with less sophisticated and cheaper equipment (>10,000€ vs. <1000€). Moreover, although according to the trading house, their detection limits are on the rage of 10^2 cell·mL^{-1}, in a study carried with YSI were only able to reach 10^3 cell·mL^{-1} [43] while any scientific approach has been found for the MS5 demonstrating its applicability to cyanobacteria detection. The photonic concentration detection system is light (500 g) and compact (180 mm x 75 mm x 106 mm) being easily portable. Additionally, the system is versatile and could be adapted

to any other bacteria by using selective labelling (HRP, AuNPs, fluorescent compounds, etc.) that could be optically detected.

6.4. CONCLUSIONS

We have presented a low-cost, easy-to-use and portable photonic prototype for the on-site detection of bacteria. The prototype integrates bacterial concentration by filtration through microfiltration membranes to deal with large sample volumes, and miniaturized optical components for in-situ fluorescence detection. The prototype testing and validations carried out using cyanobacteria as model bacteria have demonstrated an excellent correlation between the measured fluorescence and the bacterial concentrations in a range between 10^2 – 10^4 cell·mL^{-1} with a detection limit of 480 cells·mL^{-1}. Moreover, the ability of the prototype to work with volumes even at the scale of litres could allow having even lower detection limits.

The system could be adapted to the detection of different microorganisms by means of selective labelling. Thus, the prototype ability to work with large sample volumes and the versatility that can offer allows us to envision this technology as a viable and cost-effective alternative for the early warning of different target bacteria in large sample volumes.

REFERENCES

[1] M. José Figueras, J.J. Borrego, New perspectives in monitoring drinking water microbial quality, Int. J. Environ. Res. Public Health. 7 (2010) 4179–4202. https://doi.org/10.3390/ijerph7124179.

[2] Y.A. Pachepsky, A. Allende, L. Boithias, K. Cho, R. Jamieson, N. Hofstra, M. Molina, Microbial water quality: Monitoring and modeling, J. Environ. Qual. 47 (2018) 931–938. https://doi.org/10.2134/jeq2018.07.0277.

[3] European Commission, Commission regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs (Text with EEA relevance), Off. J. Eur. Union. (2005). https://www.fsai.ie/uploadedFiles/Reg2073_2005(1).pdf (accessed April 30, 2020).

[4] Commission of the European Communities, European Union. Council directive 98/83/EC on the qualityof water intended for human consumption., Off. Journalof Eur. Communities. (1998) 32–54. https://doi.org/10.1080/14683850802120702.

[5] C. Wang, F. Madiyar, C. Yu, J. Li, Detection of extremely low concentration waterborne pathogen using a multiplexing self-referencing SERS microfluidic biosensor, J. Biol. Eng. 11 (2017) 9. https://doi.org/10.1186/s13036-017-0051-x.

[6] J. Shah, S. Chemburu, E. Wilkins, I. Abdel-Hamid, Rapid Amperometric Immunoassay for Escherichia coli Based on Graphite Coated Nylon Membranes, Electroanalysis. 15 (2003) 1809–1814. https://doi.org/10.1002/elan.200302791.

[7] A. Rompré, P. Servais, J. Baudart, M.-R. De-Roubin, P. Laurent, Detection and enumeration of coliforms in drinking water: current methods and emerging approaches, J. Microbiol. Methods. 49 (2002) 31–54. https://doi.org/10.1016/S0167-7012(01)00351-7.

[8] O. Lazcka, F.J. Del Campo, F.X. Muñoz, Pathogen detection: A perspective of traditional methods and biosensors, Biosens. Bioelectron. 22 (2007) 1205–1217. https://doi.org/10.1016/j.bios.2006.06.036.

[9] EC, 2009. Council Directive 2009/54/EC of 18 June 2009 on the exploitation and marketing of natural mineral waters. Off. J. Eur. Union 164, 45–58., n.d.

[10] EC, 2006. Council Directive 2006/7/EC of 15 February 2006 concerning the man- agement of bathing water quality and repealing Directive 76/160/EEC. Off. J. Eur. Union 64, 37–51., n.d.

[11] EC, 1998. Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Off. J. Eur. Communities L330, 32–53., n.d.

[12] A.P.F. Zourob, Mohammed, Elwary, Sauna, Turner, ed., Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems, 2008. https://doi.org/10.1007/978-0-387-75113-9.

[13] J.T. Connelly, A.J. Baeumner, Biosensors for the detection of waterborne pathogens, Anal. Bioanal. Chem. 402 (2012) 117–127. https://doi.org/10.1007/s00216-011-5407-3.

[14] Y. Zhang, C. Yan, H. Yang, J. Yu, H. Wei, Rapid and selective detection of E. coli O157:H7 combining phagomagnetic separation with enzymatic colorimetry, Food Chem. 234 (2017) 332–338. https://doi.org/10.1016/j.foodchem.2017.05.013.

[15] L. Xue, L. Zheng, H. Zhang, X. Jin, J. Lin, An ultrasensitive fluorescent biosensor using high gradient magnetic separation and quantum dots for fast detection of foodborne pathogenic bacteria, Sensors Actuators, B Chem. 265 (2018) 318–325. https://doi.org/10.1016/j.snb.2018.03.014.

[16] K.D. Clark, J.A. Purslow, S.A. Pierson, O. Nacham, J.L. Anderson, Rapid preconcentration of viable bacteria using magnetic ionic liquids for PCR amplification and culture-based diagnostics, Anal. Bioanal. Chem. 409 (2017) 4983–4991. https://doi.org/10.1007/s00216-017-0439-y.

[17] W. Lee, D. Kwon, B. Chung, G.Y. Jung, A. Au, A. Folch, S. Jeon, Ultrarapid detection of pathogenic bacteria using a 3D immunomagnetic flow assay, Anal. Chem. 86 (2014) 6683–6688. https://doi.org/10.1021/ac501436d.

[18] Y. Zhang, L. Zhu, P. He, F. Zi, X. Hu, Q. Wang, Sensitive assay of Escherichia coli in food samples by microchip capillary electrophoresis based on specific aptamer binding strategy, Talanta. 197 (2019) 284–290.

https://doi.org/10.1016/j.talanta.2019.01.040.

[19] S. Podszun, P. Vulto, H. Heinz, S. Hakenberg, C. Hermann, T. Hankemeier, G.A. Urban, Enrichment of viable bacteria in a micro-volume by free-flow electrophoresis, Lab Chip. 12 (2012) 451–457. https://doi.org/10.1039/c1lc20575g.

[20] P. Ohlsson, M. Evander, K. Petersson, L. Mellhammar, A. Lehmusvuori, U. Karhunen, M. Soikkeli, T. Seppä, E. Tuunainen, A. Spangar, P. Von Lode, K. Rantakokko-Jalava, G. Otto, S. Scheding, T. Soukka, S. Wittfooth, T. Laurell, Integrated Acoustic Separation, Enrichment, and Microchip Polymerase Chain Reaction Detection of Bacteria from Blood for Rapid Sepsis Diagnostics, Anal. Chem. 88 (2016) 9403–9411. https://doi.org/10.1021/acs.analchem.6b00323.

[21] M.S. Wiederoder, S. Smith, P. Madzivhandila, D. Mager, K. Moodley, D.L. DeVoe, K.J. Land, Novel functionalities of hybrid paper-polymer centrifugal devices for assay performance enhancement, Biomicrofluidics. 11 (2017). https://doi.org/10.1063/1.5002644.

[22] X. Wu, X. Huang, Y. Zhu, J. Li, M.R. Hoffmann, Synthesis and application of superabsorbent polymer microspheres for rapid concentration and quantification of microbial pathogens in ambient water, Sep. Purif. Technol. 239 (2020) 116540. https://doi.org/10.1016/j.seppur.2020.116540.

[23] M. Martín, P. Salazar, C. Jiménez, M. Lecuona, M.J. Ramos, J. Ode, J. Alcoba, R. Roche, R. Villalonga, S. Campuzano, J.M. Pingarrón, J.L. González-Mora, Rapid Legionella pneumophila determination based on a disposable core-shell Fe<inf>3</inf>O<inf>4</inf>@poly(dopamine) magnetic nanoparticles immunoplatform, Anal. Chim. Acta. 887 (2015) 51–58. https://doi.org/10.1016/j.aca.2015.05.048.

[24] Y. Zhang, C. qing Xu, T. Guo, L. Hong, An automated bacterial concentration and recovery system for pre-enrichment required in rapid Escherichia coli detection, Sci. Rep. 8 (2018). https://doi.org/10.1038/s41598-018-35970-8.

[25] J.J. Ezenarro, N. Uria, Ó. Castillo-Fernández, N. Párraga, M. Sabrià, F.X. Muñoz Pascual, Development of an integrated method of concentration and immunodetection of bacteria, Anal. Bioanal. Chem. 410 (2018) 105–113. https://doi.org/10.1007/s00216-017-0695-x.

[26] N. Párraga-Niño, S. Quero, A. Ventós-Alfonso, N. Uria, O. Castillo-Fernandez, J.J. Ezenarro, F.X. Muñoz, M. Garcia-Nuñez, M. Sabrià, New system for the detection of Legionella pneumophila in water samples, Talanta. 189 (2018) 324–331. https://doi.org/10.1016/j.talanta.2018.07.013.

[27] J.M.E. Kasinak, B.M. Holt, M.F. Chislock, A.E. Wilson, Benchtop fluorometry of phycocyanin as a rapid approach for estimating cyanobacterial biovolume, J. Plankton Res. 37 (2014) 248–257. https://doi.org/10.1093/plankt/fbu096.

[28] F. Choo, A. Zamyadi, K. Newton, G. Newcombe, L. Bowling, R. Stuetz, R.K. Henderson, Performance evaluation of in situ fluorometers for real-time cyanobacterial monitoring, H2Open J. 1 (2018) 26–46. https://doi.org/10.2166/h2oj.2018.009.

[29] B. K. Dwivedi, Potential Risk Assessment of Cyanotoxin for Water Resources, Int. J. Curr. Microbiol. Appl. Sci. 7 (2018) 3604–3616. https://doi.org/10.20546/ijcmas.2018.702.429.

[30] H.W. Paerl, R.S. Fulton, P.H. Moisander, J. Dyble, Harmful freshwater algal blooms, with an emphasis on cyanobacteria., ScientificWorldJournal. 1 (2001) 76–113. https://doi.org/10.1100/tsw.2001.16.

[31] Environmental Protection Agency, Cyanobacteria and Cyanotoxins: Information for Drinking Water Systems, United States Environ. Prot. Agency. (2014) 1–9. https://www.epa.gov/sites/production/files/2014-08/documents/cyanobacteria_factsheet.pdf (accessed March 19, 2018).

[32] G.R. Shaw, D.P. Moore, C. Garnett, Eutrophication and Algal Blooms, Encycl. Life Support Syst. 2 (2003) 1–21. http://www.eolss.net/Sample-Chapters/C06/E6-13-04-04.pdf (accessed April 3, 2018).

[33] K.G. Sellner, G.J. Doucette, G.J. Kirkpatrick, Harmful algal blooms: Causes, impacts and detection, J. Ind. Microbiol. Biotechnol. 30 (2003) 383–406. https://doi.org/10.1007/s10295-003-0074-9.

[34] J. Chorus, Ingrid & Bartram, Toxic cyanobacteria in water. A guide to their public health consequences, monitoring, and management / edited by Ingrid Chorus and Jamie Bertram., World Heal. Organ. (1999). http://www.earthprint.com (accessed August 26, 2019).

[35] G. Persichetti, E. Viaggiu, G. Testa, R. Congestri, R. Bernini, Spectral

discrimination of planktonic cyanobacteria and microalgae based on deep UV fluorescence, Sensors Actuators, B Chem. 284 (2019) 228–235. https://doi.org/10.1016/j.snb.2018.12.111.

[36] T.- Alam, Extraction of Natural Colors from Marine Algae, J. Agric. Mar. Sci. [JAMS]. 23 (2019) 81. https://doi.org/10.24200/jams.vol23iss1pp81-91.

[37] J. Gregor, B. Maršálek, A simple in vivo fluorescence method for the selective detection and quantification of freshwater cyanobacteria and eukaryotic algae, Acta Hydrochim. Hydrobiol. 33 (2005) 142–148. https://doi.org/10.1002/aheh.200400558.

[38] A. Zamyadi, F. Choo, G. Newcombe, R. Stuetz, R.K. Henderson, A review of monitoring technologies for real-time management of cyanobacteria: Recent advances and future direction, TrAC - Trends Anal. Chem. 85 (2016) 83–96. https://doi.org/10.1016/j.trac.2016.06.023.

[39] Adel M. Mahasneh, S.M. Bdour, Microbiology Laboratory Manual, AMMAN, 2006.

[40] T.N. Ackermann, P. Giménez-Gómez, X. Muñoz-Berbel, A. Llobera, Plug and measure-a chip-to-world interface for photonic lab-on-a-chip applications, Lab Chip. 16 (2016) 3220–3226. https://doi.org/10.1039/c6lc00462h.

[41] Files · RPi · Tobias Nils Ackermann / PythonLab · GitLab, (n.d.). https://gitlab.com/TobiasNils/PythonLab/tree/RPi (accessed September 4, 2019).

[42] M.D. Pires, Evaluation of fluorometers for the in situ monitoring of chlorophyll and/or cyanobacteria, 2010. http://www.turnerdesigns.com/t2/doc/appnotes/S-0061.pdf (accessed April 20, 2020).

[43] C. Bastien, R. Cardin, É. Veilleux, C. Deblois, A. Warren, I. Laurion, Performance evaluation of phycocyanin probes for the monitoring of cyanobacteria, J. Environ. Monit. 13 (2011) 110–118. https://doi.org/10.1039/c0em00366b.

7. GENERAL DISCUSSION

GENERAL DISCUSSION

This thesis focuses on the development of integrated sensing devices for the concentration and detection of waterborne pathogens in waters. Biosensors offer the analytical rapidity needed to prevent outbreaks and the portability required for on-site detection that gold standard methods do not provide. However, whereas European Commission Directives states that at least 100 mL of water sample must be analyzed for assessing the microbiological quality of water [1–3], sensors are small and work with tiny sample volumes (≤1 mL). Consequently, they cannot match the sample volume requirements mandated by current rules (e.g. < 1 E. coli·100 mL^{-1})

Therefore, integration of concentration methods into biosensor devices is required to match sensor capacities with the actual analytical needs. Of all reported methods, membrane-based concentration techniques appear as the only method able to handle large water volumes. Hence, this thesis explores this concentration technique in the development of a platform able to process and concentrate large sample volume (100 - 200 mL) at high flow rates (30 $mL \cdot min^{-1}$). To integrate this step to a biosensing device, we have optimized an on-filter immunoassay. In this way, the filtration membrane acts as the support for both processes, concentration and detection.

For the development and test of the detection protocol and the concentration-sensing device, *E.coli* has been used as model bacteria for its fast growth and easy manipulation (**Chapter 2**). In other similar studies carried out with *E. coli,* it is demonstrated that the combination of membranes and immunoassay detection can be the right combination for bacteria detection [4,5]. However, their detection limit is still a little high (10^2 $CFU \cdot mL^{-1}$) as they continued working with small sample volumes (0.1-1 mL).

The results of the on-filter immunoassay protocol optimized in this dissertation showed that polycarbonate (PC) membranes blocked and washed with Tw-20 are the best combination to reduce the non-specific binding and detect *E. coli*. Thus, thanks to the ability to process higher sample volumes and a well-optimized detection protocol, we obtained a low detection limit of 1 $CFU \cdot mL^{-1}$.

Nevertheless, the developed concentration-detection protocol cannot be used without modifications for other microbial genera since differences in the structure and physicochemical properties of the membranes seem to affect the bacteria-membrane interactions and entrapment. Thus, the on-filter immunoassay is adapted to

Legionella concentration and detection in **Chapter 3**. Hence, whereas Tw-20 continues to be the best reagent for membrane treatment, nitrocellulose (NC) membranes are used instead of PC. Additionally, a bacterial fixation step by drying after filtration is required to obtain a good LoD of <1 CFU·mL^{-1}.

Moreover, one of the main advantages over other *Legionella* detection systems available in the market is that the monoclonal antibody employed for the immunodetection presents a good specificity and sensitivity not only for *L. penumophila* Sg.1 but also to the rest of all serogroups [6]. Consequently, our sensor offers a broader detection.

Both, *E. coli* (**Chapter 2**) and *Legionella* (**Chapter 3**) concentration-detection protocols are capable of reducing the assay times required by the gold standards from several days to 2 h. This test-time reduction is crucial and will allow taking faster preventive measures and avoid outbreaks. Moreover, the assay detects 100 CFU of *E.coli* and 70 CFU of *Legionella* corresponding to concentrations lower than 1 CFU·mL^{-1}. It is important to note that water samples of approximately 100-200 mL were analyzed in these experiments, but the platform is capable of managing volumes up to 1 L, which would allow the detection limit to be further reduced, achieving the values established by regulation (< 1 CFU·mL^{-1}).

Additionally, continuing the assays with *Legionella*, it has been shown that thanks to the reaction chamber included in the design, the whole protocol can be performed inside the concentration platform (**Chapter 3**). So, all steps of the on-filter protocol, including sample concentration, membrane blocking, antibody incubation, and washings, could be further automated by micro-pumps avoiding hand manipulation and leading to a user-friendly device. Thus, the on-filter concentration-detection protocol has been demonstrated to be a good and fast alternative to the gold standard, using less sophisticated equipment than other alternative methods such as nucleic-acid based methods.

Regarding the signal transduction, other optical biosensors have been developed for *Legionella* detection. Some of them showed a similar LoD to the obtained with our device. However, they are based on the expensive and bulky method surface plasmon resonance [7–9] (Table 7.1). Together with the optical, the transduction method most commonly integrated into biosensors is the electrochemical transduction.

Table 7.1. Optical biosensors for *Legionella* detection.

Description	**Transduction method**	**LoD**	**Ref.**
A novel high sensitive surface plasmon resonance *Legionella pneumophila* sensing platform	SPR	10 CFU·mL^{-1}	[7]
Immunosensor for detection of *Legionella pneumophila* using surface plasmon resonance	SPR	10^5 CFU·mL^{-1}	[8]
Development and application of side-polished fiber immunosensor based on surface plasmon resonance for the detection of *Legionella pneumophila* with halogens light and 850 nm-LED	SPR	10 CFU·mL^{-1}	[9]
Sub-femtomole detection of 16s rRNA from *Legionella pneumophila* using surface plasmon resonance imaging	SPRi	0.45 femtomolar 16s RNA	[10]
Rapid and multiplex detection of *Legionella*'s RNA using digital microfluidics.	Fluorescence	18 attomolar	[11]

Electrochemical sensors have simple instrumentation with high sensitivities, which makes them good candidates to obtain more compact and portable devices [12]. So, as the enzymatic substrate (TMB) used for measurements is both a chromogenic and redox substrate [13], we have attained to couple the device with an electrochemical transducer. Thus, we have used home-made screen-printed electrodes to test chronoamperometric measurements in our on-filter immunoassay obtaining an LoD of 4 CFU·mL^{-1} (**Chapter 4**). The SPE showed excellent reproducibility and low variability. Moreover, the use of cost-effective materials for their fabrication and their easy-mass production provides the possibility to dispose of the electrodes after their use, avoiding false positives by contamination between assays.

Different electrochemical sensors have been developed for *Legionella* (Table 7.2). Nevertheless, their LoDs are high. For example, similarly to our assay, Martin et al., [14] developed an amperometric magnet immunoassay. However, their detection limit was higher (10^4 CFU·mL^{-1}), and the use of magnetic particles and the sandwich configuration immunodetection using two antibodies makes the method more expensive.

Additionally, many of the developed optical and electrochemical biosensors are genosensors (Table 7.1 and 7.2) that show results in nucleic acid concentration

units, making it very difficult to establish a relationship with the number of microorganisms [10,11,15–17]. Moreover, in contrast to our on-filter protocol, the steps for nucleic acid extraction and concentration are challenging to automatize and require expensive equipment and qualified personnel.

Table 7.2. Electrochemical biosensors for *Legionella* detection.

Description	**Transduction method**	**LoD**	**Ref.**
Hairpin-DNA Probe for Enzyme-Amplified Electrochemical Detection of *Legionella pneumophila*	Columbometry	340 pM	[18]
Electrochemical nanoporous alumina membrane-based label-free DNA biosensor for the detection of *Legionella* sp	Differential pulsed voltammetry	3.1×10^{-13} M	[16]
Microelectrode array biosensor for the detection of *Legionella pneumophila*	Impedance	10^5 CFU·mL^{-1}	[19]
Rapid *Legionella pneumophila* determination based on a disposable core-shell Fe3O4@poly(dopamine) magnetic nanoparticles immunoplatform	Amperometry	10^4 CFU·mL^{-1}	[14]
Electrochemically amplified molecular beacon biosensor for ultrasensitive DNA sequence-specific detection of *Legionella* sp.	Differential pulse voltammetry	2.3×10^{-14} M	[20]
PCR-coupled electrochemical sensing of *Legionella pneumophila*	Differential pulse voltammetry	10^3-10^4 genomes	[21]

Finally, we have adapted the concentration-detection device to *Cyanobacteria* due to their capacity to produce health-damaging, and taste and odor changing compounds, in addition to its environmental impact (**Chapters 5 and 6**).

In contrast to *E.coli* and *Legionella*, cyanobacteria possess photosynthetic pigments, which gives them the characteristic to emit fluorescence. These pigments can act as intrinsic receptors absorbing light and emitting fluorescence proportional to their concentration. More concretely, the pigment phycocyanin can offer the specificity necessary to distinguish cyanobacteria from other phytoplankton species. So, the use of recognition biomolecules is unnecessary for their detection.

Different probes for cyanobacteria detection taking advantage of their fluorescence emission are already commercially available (see table 7.3). However, except for the YSI 6600 V2 and MS5 probes, the results are given in $\mu g \cdot L^{-1}$ Ch-la or $\mu g \cdot L^{-1}$ PhC of cyanobacteria, being hard to interpret (Table 7.3). The YSI 6600 V2 and MS5 probes, according to the trading house specification, have detection limits in the order of magnitude of 10^2 $cell \cdot mL^{-1}$. Nevertheless, in studies carried with YSI, the lowest concentration that has been possible to measure is one order of magnitude higher (10^3 $cell \cdot mL^{-1}$) [22], while for the MS5 probe cannot be possible to find any scientific approach employing this sensor for detecting cyanobacteria.

Table 7.3. Comparative table of commercial fluorimetric sensors for cyanobacteria detection.

Sensor	**Pigment detected**	**LoD**	**Assay time**	**Weight (Kg)**	**Price**
AlgaeTorch (bbe Moldaenke)	Total chlorophyll [µg chl-a/l] Cyanobacteria chlorophyll [µg chl-a/l]	0 - 200 µg Chl-a·L^{-1}	< 1 min	1.2	7,500€
YSI 6600 V2 with 6131 probe	Phycocyanin	220 cells·mL^{-1}	Real time	> 3	10,500€
microFLU-Blue (TriOS Optical Sensors)	Phycocyanin	0.02 µg PC·L^{-1}	Real time	0.5-0.7	6,500 €
Hydrolab Multiparameter MS5 probe (Ott)	Phycocyanin	100 cell·mL^{-1}	Real time	1.3	13,000€ -15,000€
UniLux (Chelsea Technology Groups)	Phycocyanin	<0.01 $\mu g \cdot L^{-1}$	Real time	0.100	1,400€
Our photonic device	Phycocyanin	435 cell·mL^{-1}	< 10 min		< 1,000€

We have demonstrated that a fluorescence measurement can also be integrated into our concentration-detection device. For this, we have modified the concentration platform design to incorporate fiber-optics and perform the excitation and measurement of the phycocyanin pigment present in cyanobacteria. The device has shown a limit of detection of 435 $cell \cdot mL^{-1}$. So, it is able to detect cyanobacteria in

the same order of magnitude of the commercial probes YSI 6600 V2 and MS5 with cheaper and less sophisticated equipment.

Moreover, the whole process, even when analyzing complex samples from aquaponics, takes less 10 minutes. Thus, although it is not able to give real-time results as commercial probes, it offers a fast response being able to detect concentrations even at the vigilance level established by WHO. Hence, we have been able to obtain a simple, portable, and automated photonic system for cyanobacteria bloom prevention.

In summary, the concentration-detection platform developed in this thesis offers a solution to one of the main problems faced by compact sensors when analyzing microbial quality in environmental waters, the need to analyze large volumes samples. As reported in the introduction, for the past five years, diverse concentration methods have been developed for their application in biosensors technology. However, few of them are able to operate with sample volumes of 100 mL or higher, and none of them are able to integrate this sample processing and the detection step into a single device.

In contrast, the concentration platform developed here is able to integrate large sample volume concentration and waterborne bacteria detection in a single device. Moreover, the platform is flexible since it can be modified according to the detection requirements for each bacteria. Thus, we envision that the devices developed in this thesis could be powerful tools for the detection of low pathogen concentrations in water samples.

REFERENCES:

[1] EC, 2009. Council Directive 2009/54/EC of 18 June 2009 on the exploitation and marketing of natural mineral waters. Off. J. Eur. Union 164, 45–58., n.d.

[2] EC, 2006. Council Directive 2006/7/EC of 15 February 2006 concerning the man- agement of bathing water quality and repealing Directive 76/160/EEC. Off. J. Eur. Union 64, 37–51., n.d.

[3] EC, 1998. Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Off. J. Eur. Communities L330, 32–53., n.d.

[4] I. Abdel-Hamid, D. Ivnitski, P. Atanasov, E. Wilkins, Flow-through immunofiltration assay system for rapid detection of E. coli O157:H7, Biosens. Bioelectron. 14 (1999) 309–316. https://doi.org/10.1016/S0956-5663(99)00004-4.

[5] E. Eltzov, R.S. Marks, Miniaturized Flow Stacked Immunoassay for Detecting Escherichia coli in a Single Step, Anal. Chem. 88 (2016) 6441–6449. https://doi.org/10.1021/acs.analchem.6b01034.

[6] N. Párraga-Niño, S. Quero, N. Uria, O. Castillo-Fernandez, J. Jimenez-Ezenarro, F.X. Muñoz, M. Sabrià, M. Garcia-Nuñez, Antibody test for Legionella pneumophila detection, Diagn. Microbiol. Infect. Dis. 90 (2018) 85–89. https://doi.org/10.1016/j.diagmicrobio.2017.11.005.

[7] A. Meneghello, A. Sonato, G. Ruffato, G. Zacco, F. Romanato, A novel high sensitive surface plasmon resonance Legionella pneumophila sensing platform, Sensors Actuators, B Chem. 250 (2017) 351–355. https://doi.org/10.1016/j.snb.2017.04.177.

[8] B.-K. Oh, Y.-K. Kim, W. Lee, Y.M. Bae, W.H. Lee, J.-W. Choi, Immunosensor for detection of Legionella pneumophila using surface plasmon resonance, Biosens. Bioelectron. 18 (2003) 605–611. https://doi.org/10.1016/S0956-5663(03)00032-0.

[9] H.Y. Lin, Y.C. Tsao, W.H. Tsai, Y.W. Yang, T.R. Yan, B.C. Sheu, Development and application of side-polished fiber immunosensor based on surface plasmon resonance for the detection of Legionella

pneumophila with halogens light and 850 nm-LED, Sensors Actuators, A Phys. 138 (2007) 299–305. https://doi.org/10.1016/j.sna.2007.05.015.

[10] A.M. Foudeh, J.T. Daoud, S.P. Faucher, T. Veres, M. Tabrizian, Sub-femtomole detection of 16s rRNA from Legionella pneumophila using surface plasmon resonance imaging, Biosens. Bioelectron. 52 (2014) 129–135. https://doi.org/10.1016/j.bios.2013.08.032.

[11] R. Miranda-Castro, P. De-Los-Santos-Álvarez, M.J. Lobo-Castañón, A.J. Miranda-Ordieres, P. Tuñón-Blanco, Hairpin-DNA probe for enzyme-amplified electrochemical detection of Legionella pneumophila, Anal. Chem. 79 (2007) 4050–4055. https://doi.org/10.1021/ac062260q.

[12] D.G. Rackus, M.H. Shamsi, A.R. Wheeler, Electrochemistry, biosensors and microfluidics: a convergence of fields, Chem. Soc. Rev. 44 (2015) 5320–5340. https://doi.org/10.1039/c4cs00369a.

[13] P. Fanjul-Bolado, M.B. González-García, A. Costa-García, Amperometric detection in TMB/HRP-based assays, in: Anal. Bioanal. Chem., 2005: pp. 297–302. https://doi.org/10.1007/s00216-005-3084-9.

[14] M. Martín, P. Salazar, C. Jiménez, M. Lecuona, M.J. Ramos, J. Ode, J. Alcoba, R. Roche, R. Villalonga, S. Campuzano, J.M. Pingarrón, J.L. González-Mora, Rapid Legionella pneumophila determination based on a disposable core-shell Fe<inf>3</inf>O<inf>4</inf>@poly(dopamine) magnetic nanoparticles immunoplatform, Anal. Chim. Acta. 887 (2015) 51–58. https://doi.org/10.1016/j.aca.2015.05.048.

[15] V. Rai, Y.T. Nyine, H.C. Hapuarachchi, H.M. Yap, L.C. Ng, C.S. Toh, Electrochemically amplified molecular beacon biosensor for ultrasensitive DNA sequence-specific detection of Legionella sp., Biosens. Bioelectron. 32 (2012) 133–140. https://doi.org/10.1016/j.bios.2011.11.046.

[16] V. Rai, J. Deng, C.S. Toh, Electrochemical nanoporous alumina membrane-based label-free DNA biosensor for the detection of Legionella sp, Talanta. 98 (2012) 112–117. https://doi.org/10.1016/j.talanta.2012.06.055.

[17] A.M. Foudeh, D. Brassard, M. Tabrizian, T. Veres, Rapid and multiplex

detection of Legionella's RNA using digital microfluidics, Lab Chip. 15 (2015) 1609–1618. https://doi.org/10.1039/c4lc01468e.

[18] J.T. Connelly, A.J. Baeumner, Biosensors for the detection of waterborne pathogens, Anal. Bioanal. Chem. 402 (2012) 117–127. https://doi.org/10.1007/s00216-011-5407-3.

[19] K.F. Lei, P.H.M. Leung, Microelectrode array biosensor for the detection of Legionella pneumophila, Microelectron. Eng. 91 (2012) 174–177. https://doi.org/10.1016/j.mee.2011.10.002.

[20] V. Rai, Y.T. Nyine, H.C. Hapuarachchi, H.M. Yap, L.C. Ng, C.-S. Toh, Electrochemically amplified molecular beacon biosensor for ultrasensitive DNA sequence-specific detection of Legionella sp., Biosens. Bioelectron. 32 (2012) 133–140. https://doi.org/10.1016/J.BIOS.2011.11.046.

[21] R. Miranda-Castro, N. De-los-Santos-Álvarez, M.J. Lobo-Castañón, A.J. Miranda-Ordieres, P. Tuñón-Blanco, PCR-coupled electrochemical sensing of Legionella pneumophila, Biosens. Bioelectron. 24 (2009) 2390–2396. https://doi.org/10.1016/j.bios.2008.12.014.

[22] C. Bastien, R. Cardin, É. Veilleux, C. Deblois, A. Warren, I. Laurion, Performance evaluation of phycocyanin probes for the monitoring of cyanobacteria, J. Environ. Monit. 13 (2011) 110–118. https://doi.org/10.1039/c0em00366b.

8. CONCLUSIONS

GENERAL CONCLUSION:

In this thesis, we have developed a versatile, fast, sensitive, and portable device able to concentrate and detect different bacterial contaminants in water samples. The device, made of low-cost materials, uses a microfiltration membrane to retain bacteria and is able to process large water volumes within the requirements established by the European regulation standards (>100 mL). Once microorganisms are retained on the membrane, the device is compatible with different detection and transduction methods for an on-filter detection.

The device is versatile in detecting different types of bacteria, showing limits of detection as low as 1 $CFU \cdot mL^{-1}$ and giving results in 2 h. However, its capacity to handle water volumes in the order of liters provides the possibility to lower detection limits. The device is compact, and when integrated with miniaturized equipment and adequate electronics, leads to a portable system suitable for on-site analysis.

SPECIFIC CONCLUSIONS:

1. A device for an on-filter concentration and detection of waterborne microbial contaminants has been produced and tested.
   1.1. A watertight holder fabricated in PMMA, able to process 100 mL water samples, has been designed and manufactured.
   1.2. The holder provides all the necessary fluidic components required for all concentration and detection processes.
   1.3. On-filter immunodetection of the target organisms requires the application of a membrane treatment protocol to avoid unspecific union of antibodies.
   1.4. Treatment of the microfiltration membranes with 1% Tween 20 before and after filtration, as well as washes with 0.5% Tween 20 during removal of excess antibody effectively avoids unspecific union of antibodies and minimizes the occurrence of false positives.
   1.5. The system has been tested for the detection of *Escherichia coli* and *Legionella pneumophila* in water samples. In both cases, we have determined a detection limit of 1 $CFU \cdot mL^{-1}$ and a time to detection of 2 hours.
   1.6. The assay protocol can be entirely integrated into the device, leading to a totally automated and user-friendly detection system

2. Amperometric transduction has been successfully coupled to the immunodetection protocol.
   2.1. The use of enzyme-labeled antibodies combined with the use of the redox substrate TMB generates current changes proportional to the concentration of bacteria.
   2.2. The screen-printed electrodes fabricated for carrying out amperometric measurements are low-cost, easy to mass-produce, show good reproducibility, and low variability.
   2.3. Use of a working potential of 50 mV vs. Ag and 50 s chronoamperometric measurements, the protocol is able to detected the target organisms with a LoD of 4 $CFU \cdot mL^{-1}$.

3. The on-filter concentration system has been successfully coupled to an optical detection system and used for the detection of cyanobacteria in water.
   3.1. A photonic device has been developed consisting of a polyurethane holder equipped with fluidic elements for sample filtration and optical components for sample excitation and fluorescence measurement.
   3.2. The photonic device is able to detect different concentrations of cyanobacteria with a LoD of 435 $cells \cdot mL^{-1}$.
   3.3. The device has been tested to work in real samples providing results in a few minutes (<10 min).

4. A portable photonic prototype has been manufactured for the on-site detection of bacteria.
   4.1. The miniaturized spectrometer integrated into the device allows having sensitivities as low as commercially available devices using cheaper and simpler equipment.
   4.2. The device is low-cost, portable and totally automated.

9. FURURE SCOPE

The final photonic device developed in this thesis has shown a high potential for water microbial quality control due to its capacity to process large sample volumes and reach low detection limits in addition to its simplicity and portability. Furthermore, although the device has been tested for water samples, we envision it as a powerful tool for bacterial detection in food and biological samples.

Following the results and as a future approach, the first step will be the final validation of the portable device for its application in cyanobacteria detection. Therefore, the portable device will be used to perform on-site cyanobacteria detection in real samples from different origins (rivers, small creeks, aquaponics, etc.).

Moreover, we will adapt the portable photonic device to carry out the on-filter protocol developed in this thesis to achieve a selective detection of bacteria.

Furthermore, in a future collaboration with the GTQ group from IMB-CNM, smart filters will be incorporated to the current device for early bacterial detection in water and blood (sepsis diagnostic). The filters will be modified with Prussian Blue, a metabolic indicator able to change the color when it is in contact with live bacteria, from blue to colorless. This color change will be measured by the optical fibers allowing to detect low bacterial concentrations in short time.

APPENDIX. A

COMPLEMENTARY DATA

Table A. 1 Normalized non-specific absorbance values (Abs_N) obtained for the different combinations of blocking reagent ([a] BSA, [b] WBR, [c] Tw-20 and [d] Tx-100) and washing solution concentrations and their standard deviations for each type of membrane (NC, PVDF, PC). In horizontal, washing solution concentrations ([Wash] (%)), are represented. In vertical, concentrations corresponding to blocking reagents are given (Block X%). Non-treated corresponds to maximum non-specific union assay.

a	**BSA (Abs_N)**								
	NC			**PVDF**			**PC**		
[Wash] (%)	**0.05**	**0.1**	**0.5**	**0.05**	**0.1**	**0.5**	**0.05**	**0.1**	**0.5**
Non treated	1.415 ± 0.167	1.239 ± 0.114	1.161 ± 0.058	1.671 ± 0.033	1.995 ± 0.025	1.810 ± 0.038	1.693 ± 0.248	1.628 ± 0.114	1.862 ± 0.137
Block 0.5%	1.171 ± 0.040	0.826 ± 0.227	0.278 ± 0.267	1.144 ± 0.221	0.883 ± 0.023	0.136 ± 0.028	0.162 ± 0.074	0.094 ± 0.093	0.104 ± 0.093
Block 1%	1.313 ± 0.182	0.981 ± 0.110	0.250 ± 0.043	1.441 ± 0.070	1.059 ± 0.100	0.349 ± 0.153	0.024 ± 0.099	0.069 ± 0.060	0.024 ± 0.022
Block 2.5%	1.239 ± 0.100	0.740 ± 0.095	0.207 ± 0.017	1.132 ± 0.099	0.677 ± 0.199	0.125 ± 0.084	0.026 ± 0.034	0.056 ± 0.066	0.056 ± 0.083
b	**WBR (Abs_N)**								
	NC			**PVDF**			**PC**		
[Wash] (%)	**0.05**	**0.1**	**0.5**	**0.05**	**0.1**	**0.5**	**0.05**	**0.1**	**0.5**
Non treated	1.415 ± 0.167	1.239 ± 0.114	1.161 ± 0.058	1.671 ± 0.033	1.995 ± 0.025	1.810 ± 0.038	1.693 ± 0.248	1.628 ± 0.114	1.862 ± 0.137
Block 0.5%	0.000 ± 0.018	0.000 ± 0.011	0.019 ± 0.011	0.008 ± 0.014	0.031 ± 0.022	0.022 ± 0.023	0.079 ± 0.041	0.085 ± 0.059	0.056 ± 0.059

Block 1%	0.008 ± 0.015	0.013 ± 0.017	0.021 ± 0.016	0.041 ± 0.011	0.017 ± 0.026	0± 0.016	0 ± 0.082	0 ± 0.025	0.024 ± 0.032
Block 2.5%	0.000 ± 0.006	0.013 ± 0.010	0.018 ± 0.023	0.037 ± 0.015	0.027 ± 0.022	0 ± 0.013	0.127 ± 0.182	0.037 ± 0.072	0.010 ± 0.006
c	**Tw-20 (Abs_N)**								
	NC			**PVDF**			**PC**		
[Wash] (%)	**0.05**	**0.1**	**0.5**	**0.05**	**0.1**	**0.5**	**0.05**	**0.1**	**0.5**
Non treated	1.415 ± 0.167	1.239 ± 0.114	1.161 ± 0.058	1.671 ± 0.033	1.995 ± 0.025	1.810 ± 0.038	1.693 ± 0.248	1.628 ± 0.114	1.862 ± 0.137
Block 0.1%	0.450 ± 0.033	0.375 ± 0.030	0.140 ± 0.348	1.233 ± 0.070	1.461 ± 0.037	1.282 ± 0.043	0.146 ± 0.316	0 ± 0.026	0.267 ± 0.377
Block 0.25%	0.540 ± 0.008	0.424 ± 0.070	0.304 ± 0.099	0.708 ± 0.011	0.739 ± 0.164	0.657 ± 0.064	0 ± 0.030	0 ± 0.087	0.120 ± 0.088
Block 1%	0.253 ± 0.004	0.248 ± 0.136	0.238 ± 0.034	0.225 ± 0.286	0.251 ± 0.035	0.015 ± 0.006	0 ± 0.013	0 ± 0.080	0.029 ± 0.030
d	**TX-100 (Abs_N)**								
	NC			**PVDF**			**PC**		
[Wash] (%)	**0.05**	**0.1**	**0.5**	**0.05**	**0.1**	**0.5**	**0.05**	**0.1**	**0.5**
Non treated	1.415 ± 0.167	1.239 ± 0.114	1.161 ± 0.058	1.671 ± 0.033	1.995 ± 0.025	1.810 ± 0.038	1.693 ± 0.248	1.628 ± 0.114	1.862 ± 0.137
Block 0.1%	1.316 ± 0.075	1.195 ± 0.061	0.997 ± 0.075	2.323 ± 0.081	2.053 ± 0.010	2.049 ± 0.041	2.213 ± 0.074	2.154 ± 0.123	2.166 ± 0.123
Block 0.25%	1.427 ± 0.020	1.229 ± 0.169	0.954 ± 0.178	2.195 ± 0.033	2.014 ± 0.102	1.973 ± 0.075	2.113 ± 0.129	2.237 ± 0.217	2.294 ± 0.015
Block 1%	1.425 ± 0.170	1.210 ± 0.194	1.208 ± 0.019	2.103 ± 0.130	1.998 ± 0.108	1.888 ± 0.236	2.174 ± 0.115	2.192 ± 0.127	2.227 ± 0.029

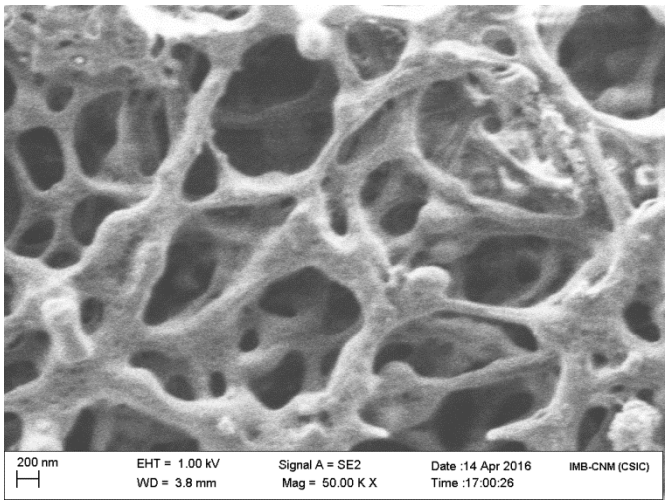

Figure A.1 Scanning electron microscope image of the Nitrocellulose (NC) membrane structure

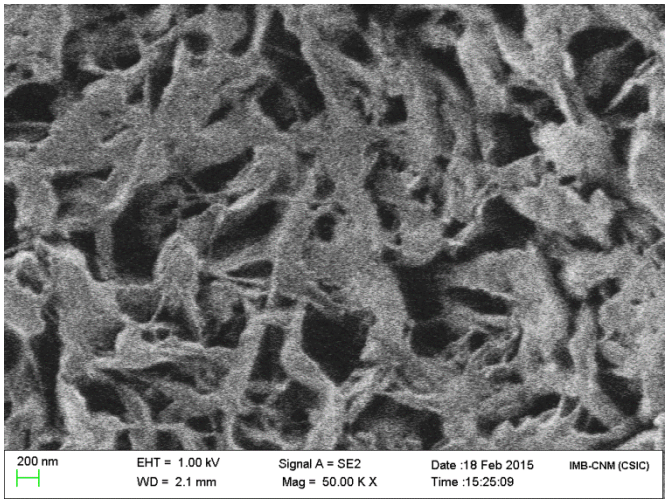

Figure A.2 Scanning electron microscope image of the Polyvinylidene difluoride (PVDF) membrane structure

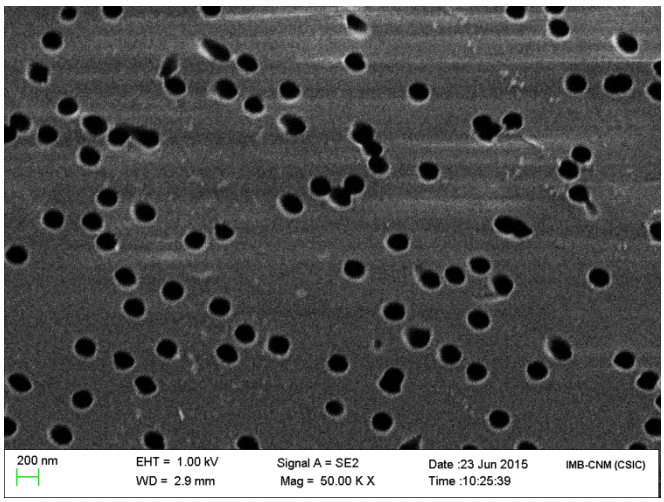

Figure A.3 Scanning electron microscope image of the Polycarbonate (PC) membrane structure

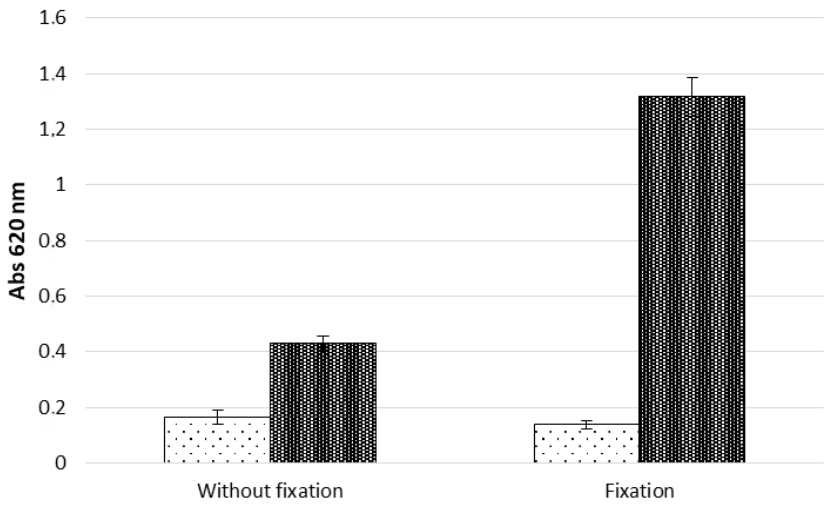

Figure A.4. Effect of fixation: NC membranes were tested with controls (white columns) and with 10^6 *L. pneumophila* CFU (shaded columns) and were tested with and without the fixation step.

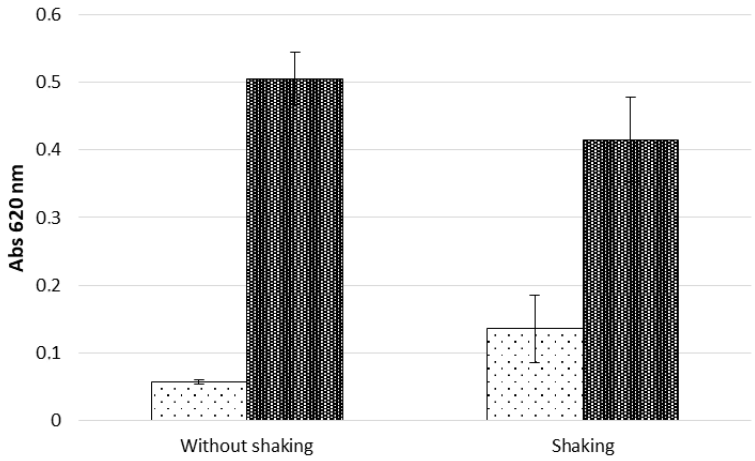

Figure A.5. Effect of shaking: NC membranes were tested with controls (white columns) and with 10^6 *L. pneumophila* CFU (shaded columns) and were tested with and without shaking.

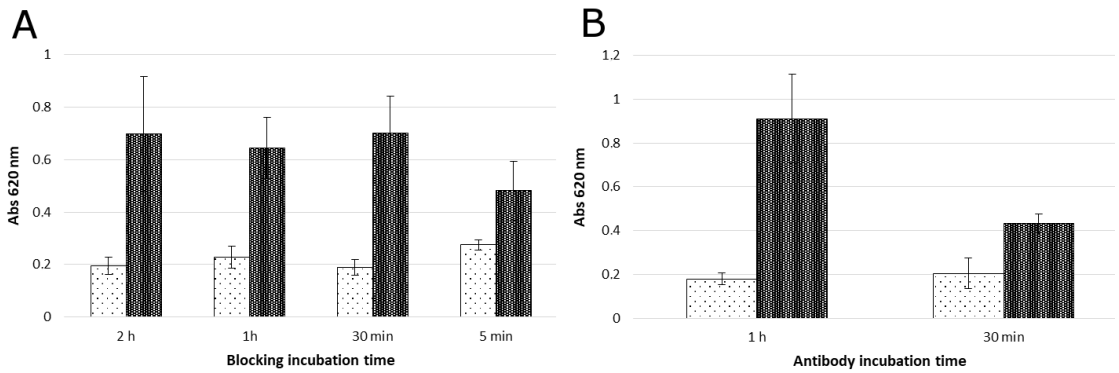

Figure A.6. Incubation time test: NC membranes were tested with controls (white columns) and with 10^6 *L. pneumophila* CFU (shaded columns) during different incubation times for the blocking incubation (A) and for antibody incubation (B).

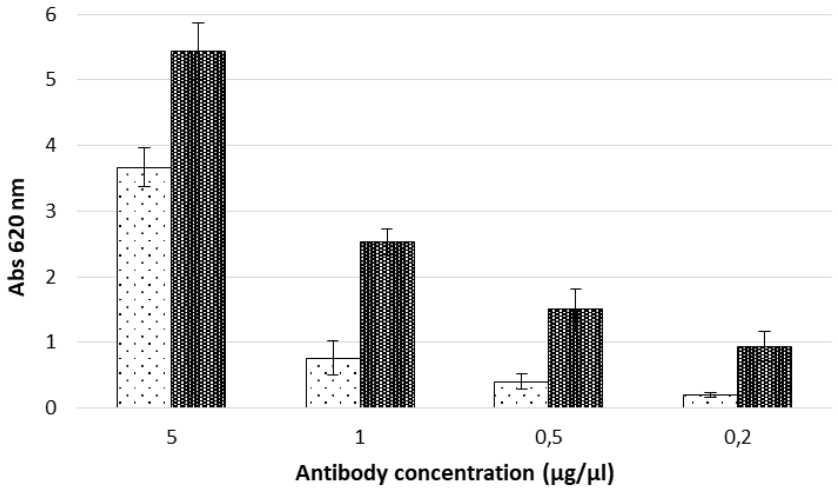

Figure A.7. Antibody concentration test: NC membranes were tested with controls (white columns) and with 10^6 *L. pneumophila* CFU (shaded columns) with different antibody concentrations.

APPENDIX. B

PUBLICATIONS

B.1 JOURNAL ARTICLES

- **J.J. Ezenarro**, N. Uria, Ó. Castillo-Fernández, N. Párraga, M. Sabrià, F.X. Muñoz Pascual, *Development of an integrated method of concentration and immunodetection of bacteria*, Anal. Bioanal. Chem. 410 (2018) 105–113.

- N. Párraga-Niño, S. Quero, A. Ventós-Alfonso, N. Uria, O. Castillo-Fernandez, **J.J. Ezenarro**, F.X. Muñoz, M. Garcia-Nuñez, M. Sabrià, *New system for the detection of Legionella pneumophila in water samples*, Talanta. 189 (2018) 324–331

- **J.J. Ezenarro**, N. Párraga-Niño, M. Sabrià, F.J. del Campo, J. Mas, F.X. Muñoz Pascual, N. Uria. Rapid detection of *Legionella pneumophila* in drinking water, based on filter immunoassay and chronoamperometric measurement. (Under review)

- **J. J Ezenarro**, T. Ackerman, P. Pelissier, D. Combot, L. Labbé, X. Muñoz-Berbel, J. Mas, J. del Campo, N. Uria. Integrated photonic system for early warning of cyanobacterial blooms in aquaponics. (Under review)

B.2 CONFERENCES

- J.J. Ezenarro, N. Uria, N. Párraga-Niño, M. Sabrià, J. Mas, F.X. Muñoz Pascual. Amperometric biosensor for rapid detection of *Legionella pneumophila* in water environments. ORAL, Ibersensor 2018 (Barcelona), Spain, 17-20 September 2018

- J.J. Ezenarro, N. Párraga-Niño, M. Sabrià, F.J. del Campo, J. Mas, F.X. Muñoz Pascual, N. Uria. Amperometric sensor for rapid detection of *Legionella pneumophilla* in fresh water. ORAL. 4th International Congress on Biosensors. (Çanakkale), Turkey, 8-11 July 2019.

- J.J Ezenarro, T. Ackerman, S. Brosel, FX. Muñoz-Pascual, J. Mas, J. del Campo, N. Uria. Label-free fluorescence cyanobacteria detection in aquaponics. POSTER. 4th International Congress on Biosensors. (Çanakkale), Turkey, 8-11 July 2019. (Best poster prize)

- J.J Ezenarro, T. Ackerman, FX. Muñoz-Pascual, J. Mas, J. del Campo, N. Uria. Fiber optics based label-free fluorescence cyanobacteria detection in aquaponics. ORAL. XXIV Transfrontier Meeting on Sensors and Biosensors (TMSB) 2019. Perpignan, France, 26th and 27th September 2019