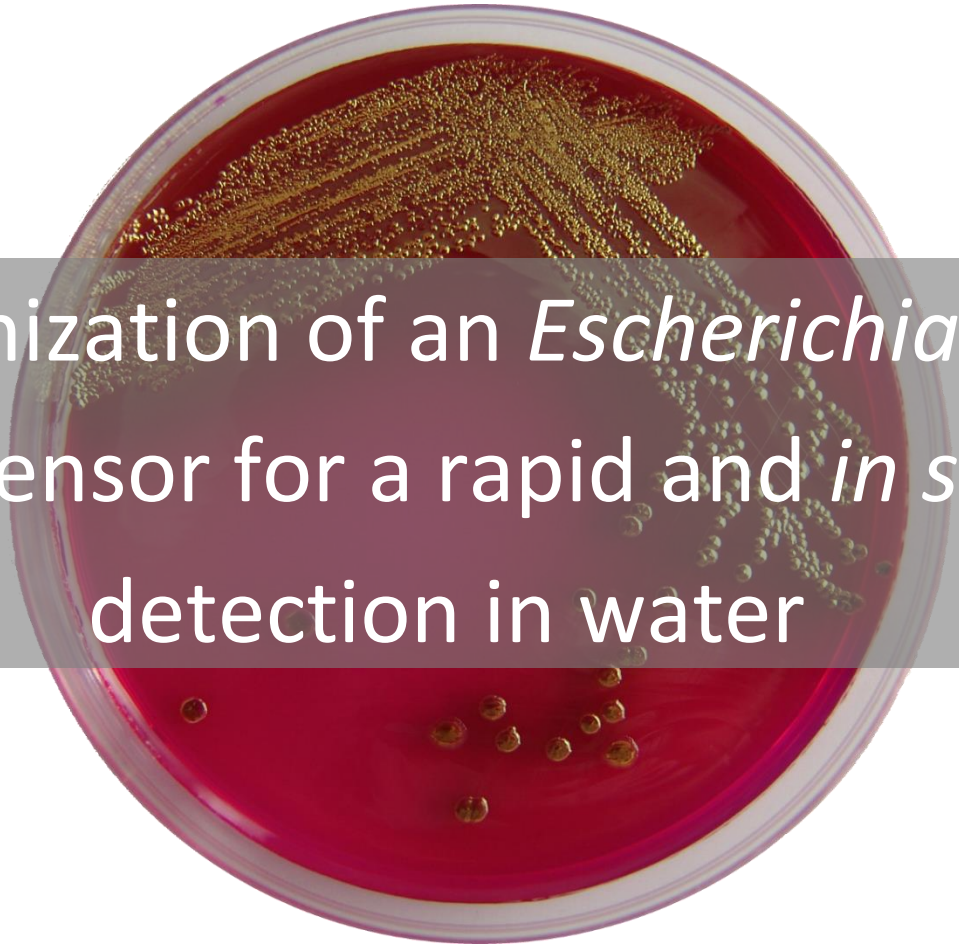
Master in Key Enabling Technologies 4 Food and + Bioprocesses



Optimization of an *Escherichia coli* biosensor for a rapid and *in situ* detection in water

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Data: 21 d'Octubre de 2016

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RESUM

Escherichia coli és un bacteri indicador de contaminació fecal i a més, alguns dels seus serotips poden ser patògens per l'ésser humà. És per això, que determinar la presència d'aquest tipus de coliform resulta vital per oferir seguretat i qualitat en el cicle integrat de l'aigua. En els últims anys no s'ha avançat gaire en les tècniques de monitorització d'aquest bacteri, ja que encara resulten cares, lentes i necessiten personal qualificat per poder utilitzar-les. El progrés dels últims anys en matèria de biosensors, ens obren un ventall de possibilitats per poder millorar el sistemes de detecció bacteriana. En aquest treball es presenta un sistema de retenció microbià integrat amb un biosensor que és capaç de detectar concentracions de fins 10^2 d'*Escherichia coli* en aigua, però que encara està en fase de desenvolupament. S'han estudiat els aspectes a millorar del sistema, com la integració del procés dins del sistema de retenció o la reducció de temps del protocol. A més, s'han considerat els aptàmers com a alternativa dels anticossos, al ser més estables. Després de les proves, s'ha pogut reduir el temps del protocol i s'han integrat certes fases dins del sistema. Algunes altres fases, no s'han pogut integrar favorablement, i es necessitaran més estudis per poder conèixer la interacció entre aquestes i el sistema. Tot i que es necessiten més assajos per que el procés sigui perfecte, s'ha avançat un pas per a que a la llarga s'obtingui un biosensor que es pugui utilitzar *in situ,* sigui sensible, ràpid i faci possible la monitorització de la qualitat de l'aigua en el sector industrial.

Paraules clau: biosensor, *Escherichia coli*¸ detecció ràpida, monitorització

RESUMEN

Escherichia coli es una bacteria indicadora de contaminación fecal y además, algunos de sus serotipos pueden ser patógenos para el ser humano. Es por eso, que medir la presencia de este tipo de coliforme, resulta vital para ofrecer seguridad y calidad en el ciclo integrado del agua. La monitorización de este tipo de organismo es muy importante, pero en los últimos años no se ha avanzado mucho en esta materia ya que las técnicas resultan caras, lentas y se necesita personal cualificado para su desarrollo. Los progresos de los últimos años en materia de biosensores abren un abanico de posibilidades para poder mejorar los sistemas detección bacteriana. En este trabajo, se presenta un sistema de retención microbiana acoplado con un biosensor, capaz de detectar concentraciones de hasta 10^2 de *E.coli,* aunque todavía está en fases de desarrollo. Se han estudiado los aspectos a mejorar del sistema, como la integración del proceso dentro del sistema de retención o la reducción del tiempo del protocolo. Además, se han considerado los aptámeros como alternativa a los anticuerpos usados, ya que resultan ser más estables. Después de las pruebas realizadas, se ha reducido el tiempo del protocolo y se han integrado ciertas fases. Algunas fases del protocolo, no han se han podido integrar favorablemente dentro del sistema, y se necesitan más estudios para poder conocer la interacción entre éstas y el sistema. Aunque se necesita más trabajo para que el proceso sea perfecto, se ha avanzado un paso para que a la larga se obtenga un biosensor que se pueda usar *in situ,* sea sensible, rápido y haga posible la monitorización de la calidad del agua en el sector industrial.

Palabras clave: biosensor, *Escherichia coli,* detección rápida, monitorización

ABSTRACT

Escherichia coli is an indicator bacterium of fecal matter in water and some serotypes can be pathogenic for humans. Quantifying the presence of this type of coliform in water is vital in order to provide safety and quality to the integrated water cycle. Although monitoring this type of microorganism is very important, in recent years there has not been much progress in this area, since analytical techniques are expensive, slow and require qualified personnel. The recent progress in biosensors has opened up a range of possibilities that can improve bacterial detection. This project presents a retention system integrated with an immuno-based biosensor that can detect a higher than 10^2 concentration of this bacterium, but it is still in development stages. Some aspects to improve the system have been studied, such as the integration of the process inside the retention system or the reduction of time. In addition, aptamers have been considered as an alternative to antibodies, as they are more stable. After the tests, the time of the protocol was reduced and certain stages were successfully integrated within the system. Some other stages though, could not be satisfactorily integrated, so further work is needed to know more about interactions. Although there's more work ahead to make the process adequate, we have made a step towards obtaining a fast, *in situ,* sensitive biosensor that can be able to monitor the water quality in the industrial sector.

Keywords: biosensor*, Escherichia coli*¸ rapid detection, monitoring

1. INTRODUCTION

Water is an important resource and its quality is the key to maintain balance in multiple biological processes. The use of water in our daily life has facilitated the creation of the integrated water cycle, which consists in the use of water resources provided by nature and its return in the best possible conditions.

One of the actual challenges that this integrated water cycle faces is pollution, including microbiological contamination.

Recorded outbreaks of microbial contamination in drinking water show that pollution by these agents and their control are major issues worldwide, as they are a huge source of infection. Only in Spain, between 1999 and 2006, 413 outbreaks were recorded involving 23642 cases [1]. It is a major concern worldwide and specially threatens the health of children as well as older and immunocompromised people.

To improve water quality avoiding contamination, depuration and purification treatments have been developed to guarantee basic public health and environmental standards [2]. In these treatments, the application of chemicals to make water potable is necessary. New proposals to make those treatments more economical and sustainable are based in the application of only the necessary amount of chemicals to treat the present pollution in water [3]. If the type and quantity of water pollutants were known it would be easier to treat and adapt the water purification process. For this reason, there's a need to study parameters that indicate water quality such as chemical and microbiological indicators. Nowadays, there are some chemical and microbiological parameters that are used as water quality indicators [4].

One of these classical indicators is *Escherichia coli,* a gram negative coliform that has been used as an indicator of fecal matter in water since the beginning of the XX century [6]. It is also the chosen indicator in the WHO Guidelines for Drinking-water Quality [4,5] and diverse countries include these bacteria in their own regulations (i.e. Europe or USA). This organism is naturally present in the lower part of the gut of warm blooded animals [7], but an overconsumption of these bacteria causes diarrhea, nauseas or headaches in humans, as well as changes in the environment. The discovery of E. Coli O157:H7, associated with hemorrhagic enteritis and hemolytic uremic syndrome has reinforced its role as an enteric pathogen in humans [8].

The detection of these bacteria in water is essential to guarantee the safety of our water treatment systems. In Europe, the control of *Escherichia coli* is mandatory for all industries involved in water treatment or reutilization (CE 2015/1787). The concentration and the frequency of the analyses differ depending of the use of water.

The classical analyses to detect *Escherichia coli* in water are based on selective culture media (multiple tube fermentation or membrane filters). These methods have several disadvantages, such as long incubation times and high interference rates with other species (6, 9). On the other hand, it has been confirmed that molecular methods, such as PCR, ISH and immunoassays are also valid. These methods permit a more rapid and sensitive analysis, but they are still expensive, require qualified personnel for

their management and are *ex-situ* [10]. It is essential for the water industry to develop a test that could be faster, *in situ* and more sensitive than the presented methods [1]. Therefore, a new concept is introduced in the development of industrial technologies: biosensing. Biosensors are analytical devices that incorporate biological material associated with a physicochemical transducer or transducing microsystem [11]. These devices are being developed for several applications, including environmental control and pollutants detection [12,13].These tools can provide fast and specific data in determined sites, where access is difficult or where *in situ* measurements are necessary. They offer the possibility of working on site, with minimal sample preparation. A wide offer of biosensors is being developed for all kinds of industries where there's a possibility of not only measuring specific chemicals, but also biological parameters and their effects [14].

1.1 Justification of the project

Considering this background, CNM-BioMEMs-TECNIO group, along with *Waterologies SL*, have patented a biosensor that could solve most of the problems of *Escherichia coli* detection in water. On one hand, they have designed a holder that makes water sample concentration easier and retains bacteria through polycarbonate membranes. Likewise, a protocol has been developed to detect and recount *E.coli* in those membranes, allowing for a cheaper, faster and *in-situ* analysis of *E. coli* concentration in water. Although the device works well, is it possible to optimize it. That's the reason why this project is focused on improving the existing protocol. ***Image 1*** shows a general summary of the desired process.

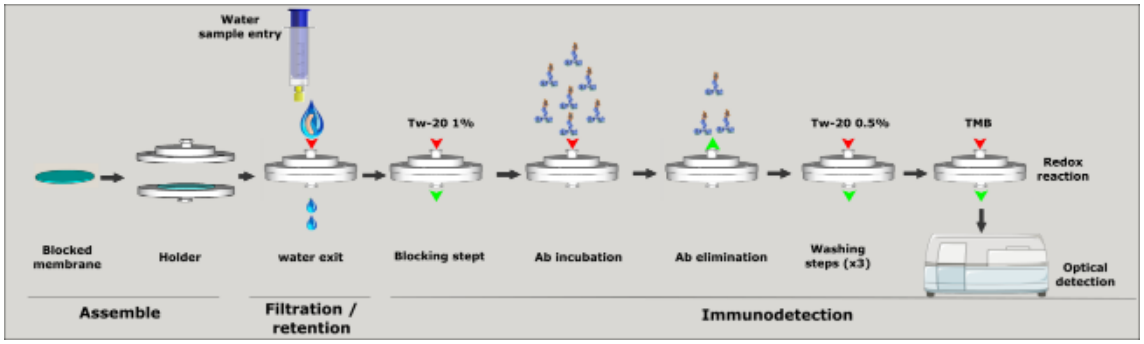


Image 1. Overview of all the protocol. Own source.

1.2 The protocol

The original protocol consisted of several stages, which had only been tested outside the designed device [15]. As well, only portions of the filter could be analyzed to show the concentration of the bacteria. In this project, those stages will be optimized and adapted for the integration inside the designed device, and the entire filter will be tested. ***Image 2*** shows an overview of all the stages we want to optimize in this project.

The protocol steps are:

1. **First membrane blocking:** treating the membrane with a detergent has been shown to decrease the posterior unspecific union between the antibodies and the membrane.
2. **Water filtration**: through a peristaltic pump the device with the blocked membrane is introduced inside the holder. The water sample is passed through the holder by silicon tubes.
3. **Second membrane blocking**: another treatment with detergent to reduce the unspecific union.
4. **Antibodies incubation**: specific antibodies that can detect bacteria are introduced on the membrane.
5. **Antibodies elimination and washing**: this step consists in washing the membrane with a low concentration of detergent and a buffer substance to remove the excess of antibodies that didn't bind with bacteria.
6. **TMB reaction**: TMB is an electron donator substance that reacts with the HRP present in the antibodies. The product that results from the reaction between the HRP and the TMB will make the solution blue. The color intensity will be directly related with the quantity of bacteria in the solution.
7. **ELISA (Enzyme-Linked Immuno Sorbent Assay)**: this scan will measure the absorbance of the solution at 620 nm. The higher the absorbance, the higher the concentration of antibody-bacteria.

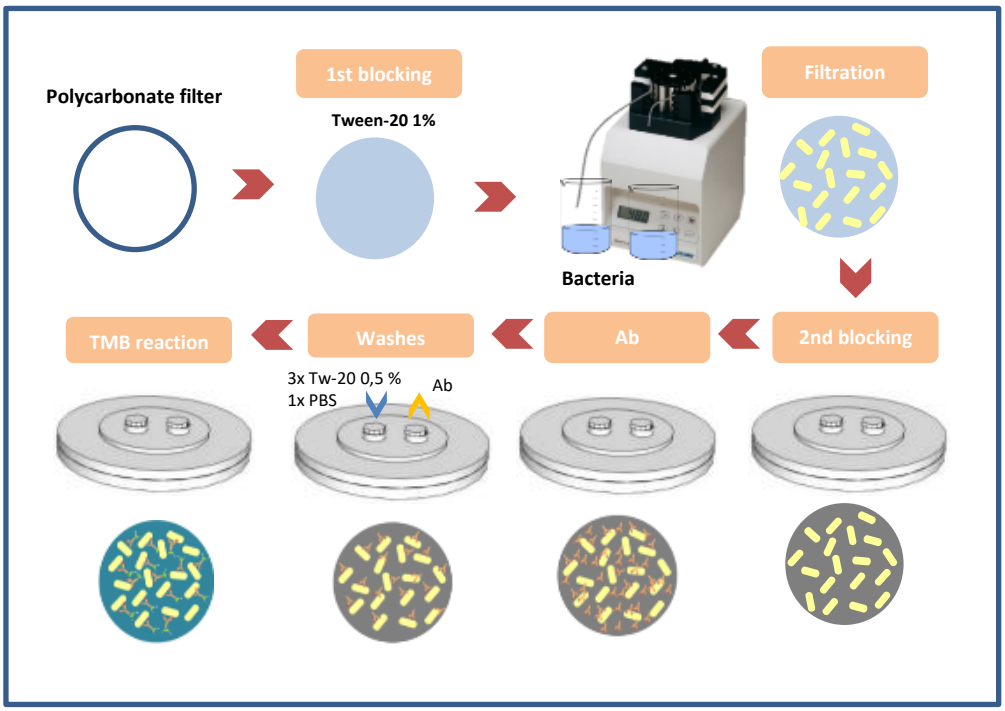


Image 2. Overview of all the protocol. Own source.

2. OBJECTIVES

The main objective is the optimization of the existing protocol [14] as well as the integration of the protocol inside the device, since only the filtration step has been included inside the holder.

a. Specific objectives

1. Reduce the time of the 1st blocking membrane treatment.
2. Adapt new conditions for the study of the whole filter. New calibration curve.
3. Integrate the protocol inside the holder:
    - **3.1** Integration of the 2nd blocking membrane treatment.
    - **3.2** Integration of the washing stages.
        - **3.2.1** Study the incidence of the flow rate on unspecific union after the 1st blocking
        - **3.2.2** Study the incidence of the flow rate on unspecific union after the antibodies incubation.
    - **3.3** Integration of the antibodies incubation inside the holder
4. Perform a preliminary study to determine if the aptamer is as effective as the antibodies.

b. Justification of the Objectives

1. Reduce the time of the 1st blocking membrane treatment.

In the previous protocol, the treatment of the membrane lasted for 2 hours, as there was no bibliography of how the detergent could affect the permeability of the membrane versus the antibodies. As one of the proposals for the improvement of the protocol was time reduction, there's a need to know if time is a relevant condition in the treatment.

2. Adapt new conditions for the study of the whole filter. New calibration curve.

The study of the concentration of bacteria in the previous protocol was made through the perforation of the membrane itself. Four pieces of 5 mm each were analyzed in the ELISA reader. This condition was considered a reason for the improvement, since the exact amount of bacteria was a mere approximation. That's why, that the analysis of the entire membrane was vital to perform a more accurate test.

To do this, cellular culture plates are going to be used and conditions such as the amount of antibody and TMB are going to be recalculated. After the adaptation of these new conditions, a new calibration curve is going to be calculated.

3. Integration of the protocol inside the holder

The integration of the protocol inside the holder is crucial, since the main purpose of this project is to accomplish a measure that can be *in situ (*without moving the sample) in the shortest possible time. The study of the integration inside the holder has been divided into several assays: each stage of the protocol was compared inside and outside the holder.

3.1 Integration of the 2nd blocking membrane treatment.

The 2nd membrane treatment will be performed inside and outside the holder to see if the amount of blocking agent that fills the holder is enough to block the membrane.

3.2 Integration of the washing stages.

To know if the washes could be integrated, the study of the incidence of the flow rate on the membrane is executed after the 1st blocking stage and after the 2nd blocking stage. We want to know if the flow rate can drag part of the blocking, antibodies or bacteria.

3.3 Integration of the antibodies incubation inside the holder

First of all, the concentration of antibodies will be recalculated for the incubation inside the holder, as the maximum volume of the holder is of 700 µl. After that we will compare if the incubation inside and outside the holder are similar.

4 Preliminary study of the aptamer

Aptamers are a sequence of nucleic acid molecules that are starting to be investigated for their clinical use as they are equivalent to antibodies. They are smaller, highly specific and they are not as temperature sensible as antibodies [16]. As in some studies has been a good tool to attach to *Escherichia coli* [17], we'll study if they can work in our present conditions.

3. MATERIALS

- **The holder**

Two methacrylate pieces form the holder, bound by 4 screws. In the upper piece, we'll find the reaction chamber (20 mm of diameter) and a circular slot where a 3 mm O-ring will be placed to ensure the enclosure is airtight. The input and output channels are also located in the upper part, where the water and the necessary reactives will be pumped ***(Image 3).***

The lower piece is flat and the evacuation channels assure internal pressure regulation. The circular polycarbonate membrane is placed between both parts.

The total diameter of the holder is 42 mm and all the connectors of the system are Luer type, standard for small scale fluidic systems.

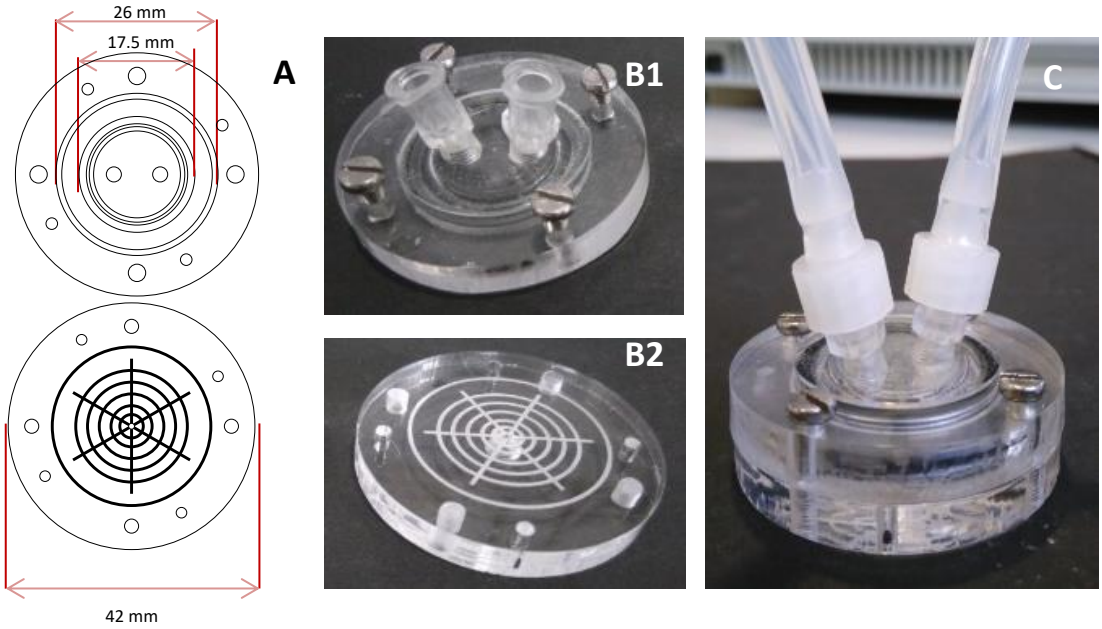


Image 3. The proportions of the holder (a), the superior and the inferior methacrylate pieces (b1/b2) and the Luer connectors attached to the holder (c). Own source.

- **The membranes**

The membranes are the physical barriers that permit the retention of *Escherichia coli* from water. The chosen membrane is a polycarbonate filter that has a 0.2 nm pore size (Whatman Nuclepore Track Etched Polycarbonate, GE Healthcare Life Science). The shape of these pores and the membrane mesh retain bacteria. The filter size is 25 mm in diameter ***(Image 4)***. In previous studies, the evaluation of possible materials was made, and the conclusion was that polycarbonate filters of 2 nm pores are the best to retain *Escherichia coli* [15].

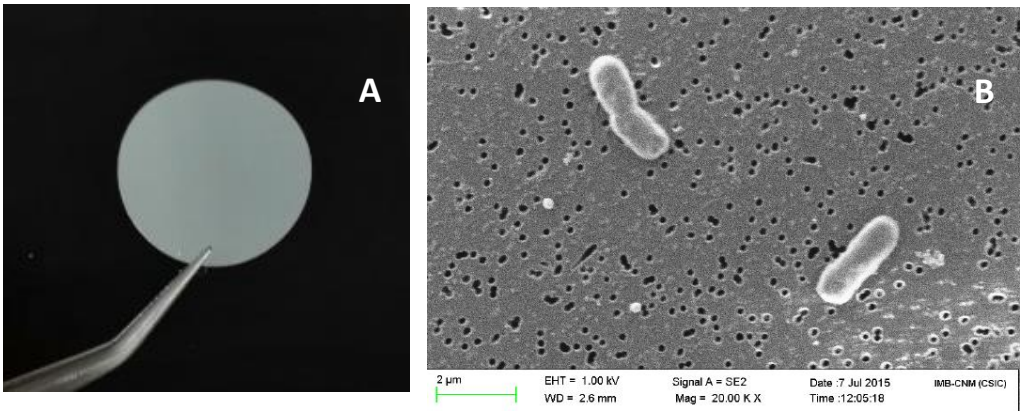


Image 4. The polycarbonate membrane (a). SEM view of the membrane with *E.coli* retained in the pores (b). Own source.

- **Bacteria**

The chosen strain to make the antibody assays was *Escherichia coli* (ATCC 10538) from American type culture Collection. To perform the aptamers tests, another strain of bacteria was used *Escherichia coli* (ATCC 17036). To prepare the desired concentration, the bacteria suspension were grown overnight at 37 ºC in a Luria-Bertrani medium. The concentration of the culture was calculated with the plate counting method in Luria Bertrani Agar Plates (Sigma Aldrich). They were left to grow overnight at 37 ºC. The preparation of the overnight culture and the procedure of serial dilutions were always made in sterile conditions.

- **Reactives**

  - **Tween-20 (Polyethylene glycol sorbitan monolaurate):** this substance is a non-ionic detergent that prevents the unspecific union of antibodies with the membrane). The membranes we'll be treated twice, once before the filtration and once after the filtration, with 2 ml of Tween-20 at 1% concentration. At a lower concentration and agitation at 40 rpm (Mini orbital shaker SSM1, Stuart), the detergent removes the antibodies that didn't bind with the membrane or the bacteria [15]. That's the reason

why, after the antibodies incubation, membranes are washed 3 times with the Tween-20 at 0.5% concentration for five minutes. By applying those treatments to the membranes we eliminate false positives from the final result, as only the antibodies bounded with bacteria will remain The different concentrations of Tween-20 were prepared with 0.01 M PBS (Phosphate Buffered Saline, Sigma Aldrich).

- **PBS (Phosphate Buffered saline, Sigma Aldrich)**: buffer solution commonly used in biological research, as the osmolality and the present ions stabilize cell solutions. The application of 2 ml of PBS is the last washing step.

- **Antibodies**

Anti-*E.coli* antibodies (US Biological) were used in a concentration of 1:100 (20 µl/1980 µl PBS). The binding between bacteria and antibodies requires 30 minutes and a low 30 rpm rotation.

- **Aptamers**

The chosen aptamers for the test were specific for *Escherichia coli* 17036 strain. Aptamers need a previous incubation with HRP because they don't form a natural complex [16]. The binding between the aptamer and HRP requires of 30 minutes at low rotation (30 rpm). After that, the solution of aptamer and HRP is mixed with PBS (40 µl for 1960 µl of PBS), and the membrane is incubated with the solution for another extra 30 minutes.

- **Peristaltic pumps**

Two peristaltic pumps were used for different tests. For water filtration, the Gilson Minipuls 3® peristaltic pump was utilized, as it works at high pressures and flow rates (0.5 ml/s).

For the washing tests, as low flow rates were needed, the pump was a piezoelectric pump (ThinXXS 5V) that works with pulses.

Silicon tubes (Gilson, 3.18 mm) tubes were used to make the liquids circulate through the pumps. To remove the residue that could remain inside the tubes in each assay, they were cleaned every time with bleach reduced with water ***(Image 5)***

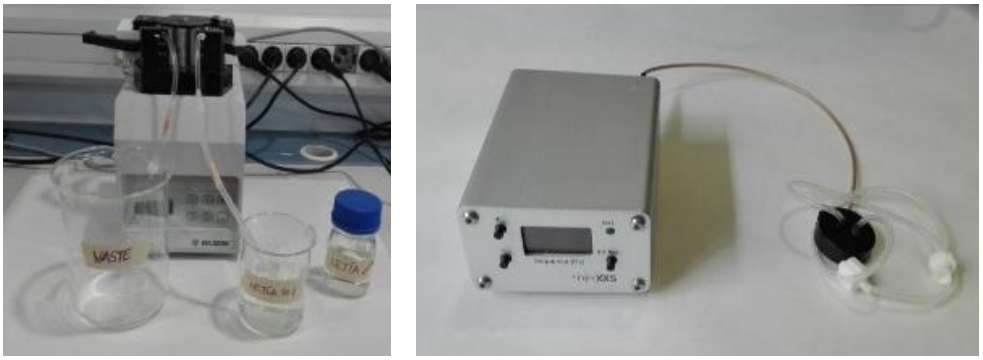


Image 5. Gilson Minipuls 3® (a) and piezoelectric pump (b). Own source.

- **TMB**

3, 3', 5, 5'-Tetramethylbenzidine or TMB is a chromogenic substrate used for staining procedures in enzyme-linked immunosorbent assays (ELISA). The oxidation of this compound (from 3,3',5,5'-tetramethylbenzidine (TMB) to 3,3',5,5'-tetramethylbenzidine diimine), changes the color of the solution into a bluish tone that can be read in the spectrophotometer or the ELISA reader. TMB reacts to the HRP molecule, present in antibodies [18].

- **Plates**

The plates used in this study are cell culture plates, for blocking membranes, antibodies incubation and washes and 96-wells plates (Nunc-Immuni MicroWell 96 wells, Sigma-Aldrich) to read absorbance in the ELISA reader (Multiskan Ex, Thermo Scientific) *(Image 6)*

Image 6. Cell culture plates and ELiSA 96-wells plate. Own source.

- **ELISA microplate reader** (Multiskan Ex, Thermo Scientific).

This instrument is used to read absorbance from samples placed in microplates. A light bulb illuminates the microplate, using a specific wavelength (filtered by a monochromator), and the light detector located in the opposite site will measure how much of the initial light is transmitted through the sample. The received transmitted light will be directly related with the concentration of the molecule. In this project the selected wavelength is 620 nm, and the volume of the samples in each well is of 100 µl. After the reading, a specific software will transform the data into an .exe file, where the data can be processed. In ***Image 7*** we can see all the system (reader and computer).

Image 7. ELISA reader (left) and attached computer for performing the reading. Own source.

4. METHODS

a. Reducing the time of the first membrane blocking

In the previous protocol, the first membrane blocking was performed within 2 hours. The objective of this assay is to know which is the minimum and most suitable time to let the detergent (Tween-20, 1%) reduce the unspecific bounding of antibodies.

Six different conditions were established. In one cell culture plate 6 membranes were placed: one blank (no Ab+Tw-20 for 2 hours), one with maximum unspecific binding (Ab+no Tw-20), one incubated with the Tw-20 for 2 hours, one incubated for 1 hour, one incubated for 30 minutes, one incubated for 15 minutes and one incubated for 5 minutes. All the membranes that were incubated with the detergent had the same amount and concentration of detergent: 2 ml of Tween-20 at 1%.

Later, all the membranes (except for the blank), were incubated with the antibody (anti *Escherichia coli*, USBiological) for 30 minutes at 30 rpm with a 1:100 (20 µl Ab/1980 PBS concentration.

To remove the antibody that didn't bind with the membrane, the membranes were washed three times with 2 ml of Tween 0.5% for 5 minutes, placing them in a new plate each time (to avoid dragging unbound antibodies). Another extra wash was performed, but this time with PBS 1% (Phosphate Buffered Saline, Sigma) for 5 minutes.

Once the membranes are washed, they are introduced in a new culture cell plate and immersed in 500 µl of TMB for 16 minutes at 40 rpm, so the reaction can be placed. Once the time has passed, 100 µl of each membrane is extracted and placed in an ELISA 96-wells plate (Nunc-Immuni MicroWell 96 well, Sigma-Aldrich) with 3 replicas. Then, the 96-wells ELISA plate is introduced inside the ELISA reader and the software is started (Multiskan Ex, Thermo Scientific) to make a punctual read at 620 nm.

b. Adapt new conditions for the study of the whole filter. New calibration curve.

Previously, the detection of the bacteria was made through the perforation of the membranes [15].One of the proposals was to do the detection on the full membrane, so the measure could be more exact and reliable. To do this, culture cell plates were used as physical bases of all the protocol. The concentration of detergents and antibodies were adapted for the new conditions.

First of all, 10^3,10^4,10^5,10^6 concentrations of *Escherichia coli* in 100 ml of distillated water were prepared (three replicas of each concentration). The samples were prepared after serial dilutions from the overnight culture at 37ºC.

After that, 15 membranes polycarbonate were blocked with Tween-20 at 1% for 20 minutes at 40 rpm. Later, they were placed in a dry cell culture plate and introduced one by one in the holder.

Once the membrane is located inside the holder, the tubes are connected to the device and the peristaltic pump is initialized. The 100 ml with diluted bacteria (except for the blank) is pumped inside the device with a 0.5 ml/s flow rate.

After filtration, the membrane is placed in another dry cell culture plate and the antibody is inoculated inside (20 µl in 2 ml of PBS). The antibody incubation lasts for 30 minutes at 40 rpm (to make sure the antibodies bind correctly).

Afterwards, the second blocking treatment of the membrane is done (15 minutes, 40 rpm with Tween-20 at 1%), so we reduce the unspecific bounding of the antibody.

Subsequently, the membranes are washed three times with Tween-20 at 0.5% and once with PBS for 5 minutes.

Once the membranes have been washed, they are introduced in a new culture cell plate and immersed in 500 microliters of TMB [15] for 13 minutes at 40 rpm, so the reaction takes place.

Once the time has passed, 100 µl of each membrane are extracted and placed in the ELISA 96-wells plate [15]. Then, the 96-wells ELISA plate is introduced inside the ELISA reader and the software is started (Multiskan Ex, Thermo Scientific) to make a punctual read at 620 nm.

4.3 Integration of the protocol inside the holder:

The integration of the protocol inside the holder is crucial, since the main purpose of this project is to accomplish a measure that can be *in situ* (without moving the sample) in the shortest possible time. The study of the integration inside the holder has been divided into several assays: each stage of the protocol was compared inside and outside the holder.

4.3.1 2nd blocking treatment inside the holder

To study if the second treatment of the membrane could be integrated inside the protocol, we followed the same steps described in the previous section except for the one we wanted to study (second treatment).

Eighteen membranes were treated (9 outside the holder, 9 inside), 6 blank samples, 6 10^4 samples and 6 10^6 were diluted in 100 ml of distilled water. Once the water filtration was performed, the membranes that were treated outside followed the classical protocol (20 minutes, Tween-20 1% at 40 rpm). The membranes that were tested inside the holder were immerged in Tween-20 at the same concentration for the same time (15 minutes) covering the bottom of the holder with an output Luer plug ***(Image 8*)**. The only condition that differed was the used volume, 2 ml for the outside treated membranes and 700 µl for the inside treated membranes.

After that, the same conditions as the previous section were performed (antibody incubation, washes and detection).

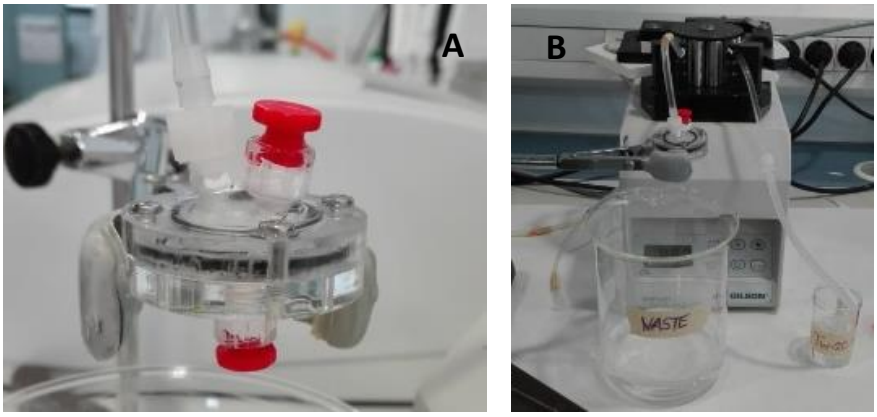


Image 8. Holder with the attached Luer plugs to retain Tw-20 (a), image of the whole system (b). Own source.

4.3.2 Integration of the washing stages

a. Study the incidence of the water flow rate on the amount of Tween-20 present in the membrane

The first step to know if the washes can be introduced inside the holder is to know if the water at a certain flow rate can affect the 1st blocking of the membrane by dragging part of the present detergent. To do this, we have selected 6 different water flow rates (0.25, 0.5,1, 1.25, 1.5 and 1.75 ml/min) pumped by a piezoelectric pump with silicon tubes.

21 membranes have been blocked with Tween-20 1% during 10 minutes outside the holder. 18 of them have been inserted inside the holder, and one by one were tested with a determined flow rate with distillated water. Each flow rate was tested with 3 membranes (3 membranes for 0.25 ml/min, 3 for 0.5 ml/min etc.) All of the treatments had the same duration: 5 minutes. Besides 3 more membranes were considered blank, therefore none of those were treated with water.

Once all the membranes have been treated, the incubation of the antibodies in each of them was started. After that, the washing stages and detection were the same described in the previous section. The 2nd blocking treatment wasn't performed as we wanted to know the incidence only in the 1st blocking.

b. Study the incidence of the flow rate on the antibodies

To develop this study, all phases of the protocol were performed outside the holder, except for the filtration and the washings. First of all, 9 membranes were blocked for 10 minutes with Tween-20 1%.

Then the incubation of the antibody was performed following the original protocol (30 minutes at low rotation).
Subsequently, 3 of the membranes were washed following the original protocol (outside the holder).

By the other side, the remaining 6 membranes were introduced inside the holder, were the washing solution was pumped by the peristaltic pumps at a flow rate of 1 ml/min for the first 3 membranes and 0.5 ml/min for the last three. The washing solutions was Tween-20 at 0.5 % (repeated 3 times) for 5 minutes each treatment. After that, the membranes are removed outside the holder and the last PBS treatment is performed. Finally, all the membranes were moved to dry cell culture plates, where the incubation with TMB was performed for 16 minutes. The solutions were analyzed by the ELISA reader.

4.3.3 Integration of the antibody incubation

After studying the integration of the previous stages, the next step was to integrate the incubation of antibodies inside the holder.

3 membranes were blocked for 10 minutes with Tween-20 at 1% outside the holder, at a low rotation of 30 rpm. After that, the membranes were introduced inside the holder, were 100 ml of distillated water was pumped following the steps described before. Subsequently, the 2nd blocking of the membrane was performed, pumping 700 µl of Tween-20 at 1% inside the holder, and leaving it for 15 minutes (as the assay described in the previous section).

Once the membrane was treated with the detergent, the antibodies were pumped inside. The amount of antibodies was modified, as the total volume of the holder differs from the culture cell plates (7 µl of Ab in 693 µl of PBS). The solution was left for 30 minutes inside the holder.

To perform the next stages, the 2nd superior slot was opened and connected to another silicon tube, so the exit fluid could be evacuated from the holder. After that, the washing solutions were pumped inside the holder for five minutes each (3 washes of Tween-20 at 0.5% and 1 wash of PBS). Finally, 500 µl of TMB was introduced inside the holder, and after 16 minutes of reaction, 200 µl of the liquid was extracted to read the absorbance at 620 nm in the ELISA reader.

4.4 Perform a preliminary study with the aptamer.

First of all the complex aptamer-HRP was prepared, before the treatment as they don't form a natural complex. 20 µl of aptamer and 20µl of HRP were inoculated in 1960 µl of PBS in a well of a cell culture plate (one well for each membrane used). Then, the incubation lasted 30 minutes for the aptamer and HRP to bind, at a low rotation of 30 rpm.

At the same time, 2 membranes were blocked following the steps described previously. After that, one by one were introduced inside the holder: one was treated with 100 ml of pure water (blank), and the other one with 10^6 of *E.coli 17076* (in 100 ml of distillated water). After the filtration, both of the membranes were introduced in dry wells, and inoculated with the aptamer-HRP complex prepared before.

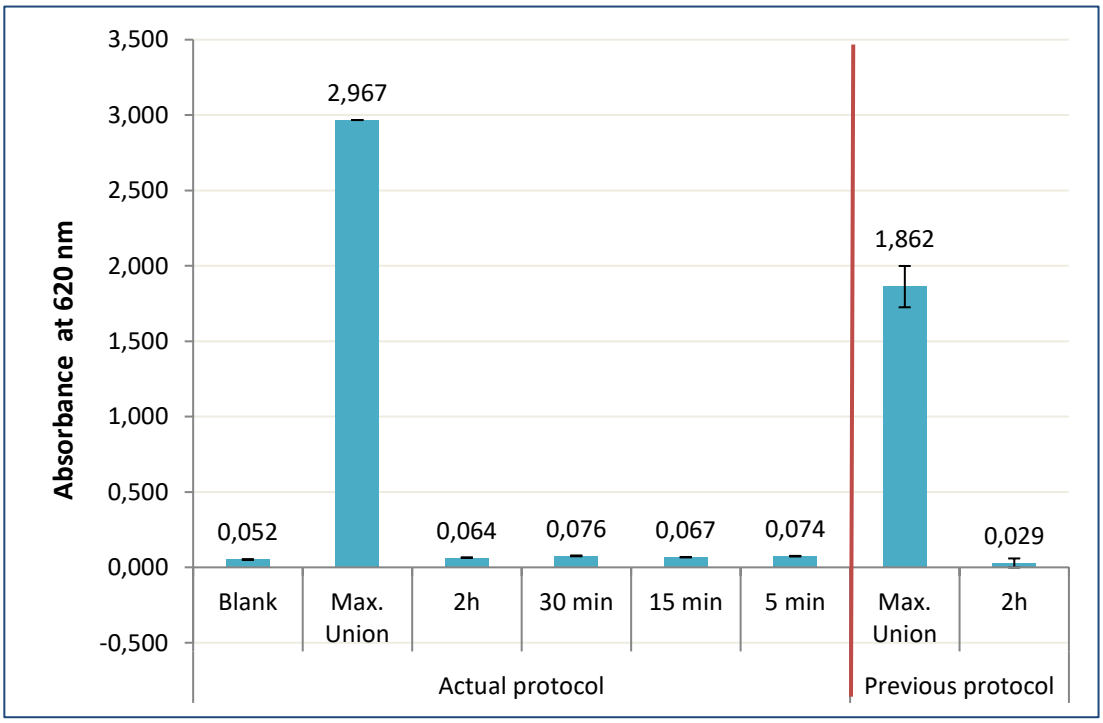
After that, the washing stages and detection were the same described in the previous sections.

5. RESULTS AND DISCUSSION

5.1 Reducing the time of the first membrane blocking

In ***Graphic 1*** the results of the assay are displayed and as well compared with the results in the previous protocol (after treating the membrane for 2 hours).

In the ***Graphic 1*** we can observe that the membrane that had no detergent, showed more absorbance, so more antibodies were attached to the membrane (maximum unspecific union). On the other side, the blank membrane (no antibodies) showed the minimum absorbance. The membranes that differed on the time of incubation with the detergent showed similar results. After these results, we can tell that the amount of time of the membrane 1st blocking treatment t is not a condition that modifies the union between the antibody and the membrane (unspecific union). From now on the minimum pretreatment time (5 minutes) will be included in the protocol, because it showed to be as much as effective as treating the membrane for 2 hours.



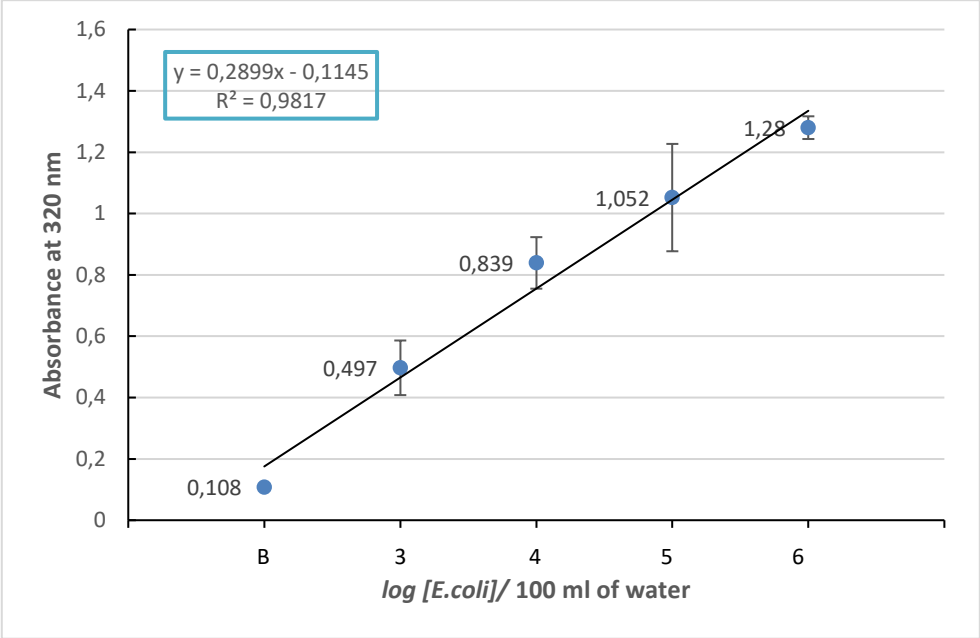
Graphic 1.. Absorbance in the different membranes with different times of blocking.

5.2 Adapt new conditions for the study of the whole filter. New calibration curve.

After adapting the new conditions for the study with the whole filter (amount of antibodies and TMB), a new calibration curve was performed to see if the protocol was still valid for quantifying the amount of *E.coli* in water.

In ***Graphic 2*** is displayed the results of the study with the whole filter, as we can see, as the concentration of bacteria increases, the absorbance increases as well. We can see that analyzing the whole membrane using cell culture plates (without perforating the membrane, as before) is more accurate and still valid to determine the amount of bacteria present.

This data shows a linearity of 0.98 R^2, making us conclude that the protocol is still valid for *Escherichia coli* detection.



Graphic 2. Absorbance in the different membranes with different concentrations of *E.coli*

5.3 Integrate the protocol inside the holder

5.3.1 Integration 2nd blocking inside the holder.

In this study, we've compared the effectiveness of performing the 2nd blocking treatment inside vs outside the holder, with blank dilutions, 10^4 and 10^6 concentrations. The differences between blocking outside and inside was the volume used (2 ml outside and 700 µl inside).

In ***Figure 1***, the two graphs obtained are shown. In ***Graphic A*** there's described the results following the original protocol (2nd blocking outside the holder) and in ***Graphic B***, the results of integrating the treatment inside the holder. As we see, both graphs show a positive trend, by increasing the concentration, the absorbance increases as well. On the other hand, the equation of the inside results, shows a higher offset by relating it to the outside results. This results could make us think that the part of the membrane that touches the O-Ring doesn`t get wet, therefore, the antibodies have higher affinity in that region (Image). By looking the slopes of both trends, we see that the equations are not parallel. The equation of the original protocol has a higher slope than the equation of the integrated membranes. This difference could be result of the amount of Tween-20 used. In the outside protocol more detergent was used (2 ml) than in the integrated protocol (700 µl). Furthermore, it is observed that the results of the integrated within the holder membranes are highly variable (large standard deviations). This could have to do with the holder material, though further work is needed.

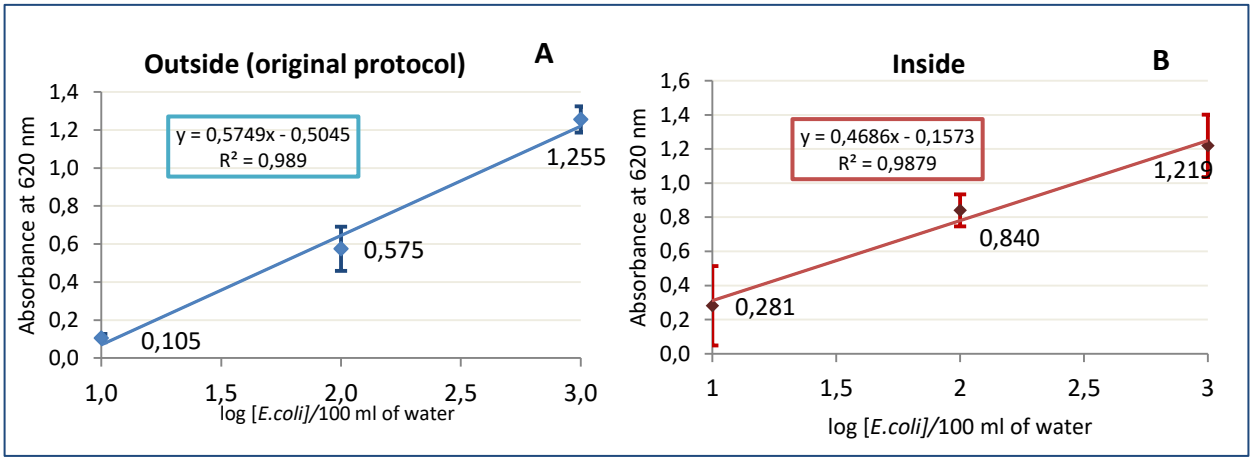


Figure 1. Absorbance in of the membranes, by following the original protocol (A) or blocking inside the holder (B)

5.3.2 Integration of the washes

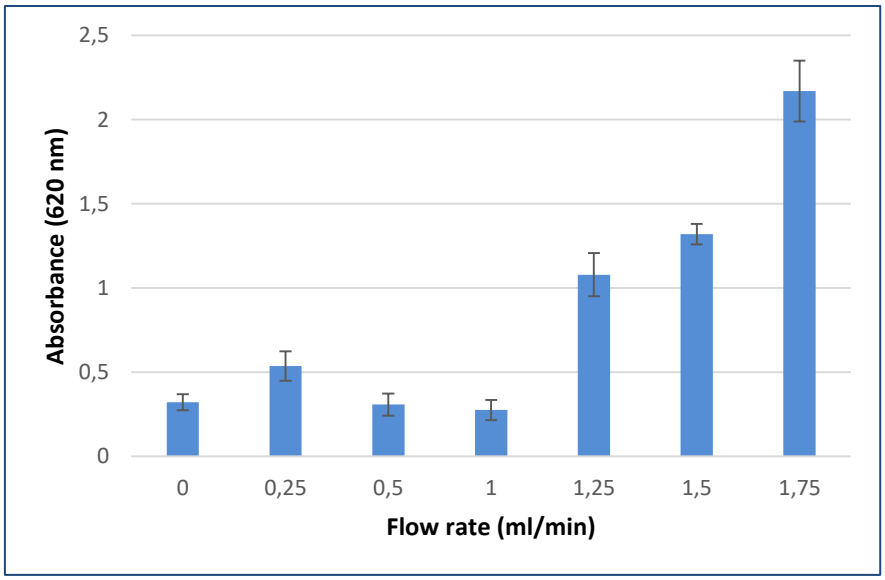
a. Study the incidence of the water flow rate on the amount of Tw-20 present in the membrane

Graphic 4 shows the amount of detergent left in the membrane in comparison with the water flow that has been subjected to the membrane. As more absorbance, less detergent.

As we can see in the graph, at higher flow rates (1.25, 1.5 and 1.75), more detergent is dragged out of the membrane, increasing the unspecific union of the antibodies. In the other membranes, we can see that the flow rate affects differently to the amount of Tween-20 left. At 0.25 ml/min, part of the detergent has been more diluted than higher flow rates (0.5 and 1). One explanation could be the laminar flow created by the low speed of the pump: the water flows in parallel layers, with no crossed currents and the particles of the fluid move in straight lines parallel to the membrane [19]. In our case, more water particles get in contact with detergent particles, and subsequently there is more dilution of Tween-20.

The membrane that has not been subjected to any flow (didn't lose any of the Tween-20) has a low absorbance, because the antibodies had a low affinity with the membrane as it was blocked by the detergent.

Flow rate 0.5 ml/min and 1 ml/min showed to be the ideal rates as the water didn't remove any of the detergent (low antibody binding).

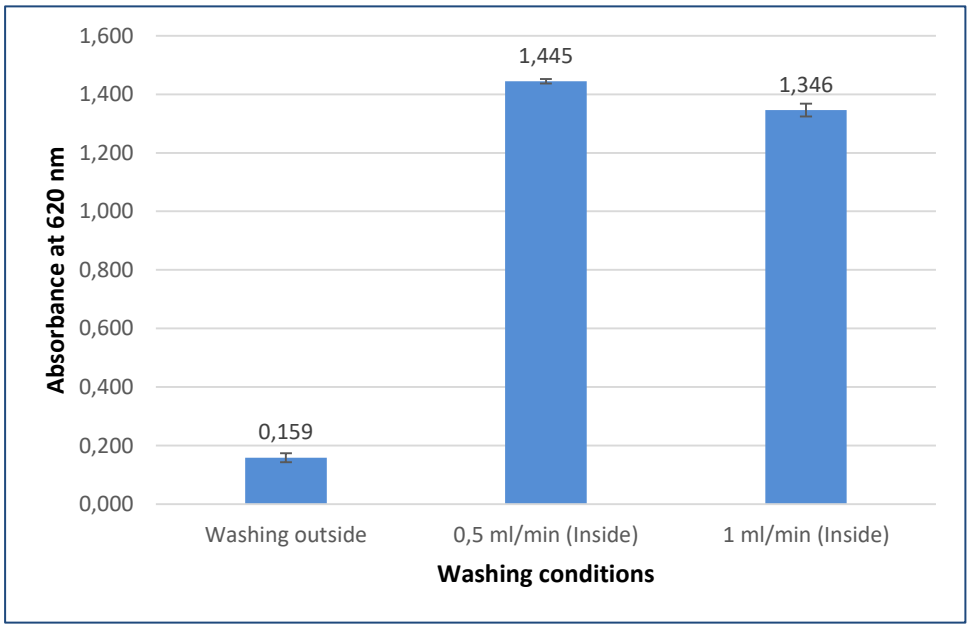


Graphic 4. Absorbance in the different membranes treated with different flow rates

b. Study if the flow rate affects unspecific union of the 2nd blocking.

In *Graphic 5* we can observe the differences between washing the membranes with Tween-20 0.5% inside the holder at different flow rates and washing them outside (following the original protocol). We've chosen 0.5 ml/min and 1 ml/min flow rates, as in the previous section, showed to be the conditions that affected less to the amount of detergent in the membrane.

We have to consider, that in this assay, the 2nd blocking treatment wasn't performed, and subsequently, the unspecific binding should be higher. However, as we observe in the graph, performing the washes inside the holder, didn't work as the unspecific binding turns out to be higher. This means that antibodies weren't separated from the membrane successfully. The washes inside the holder were made following the same conditions (3 washes at 5 minutes each) and a greater amount of detergent was used. This may suggest that the design or the material of the holder interferes with the washing processes, making them ineffective when they are integrated.



Graphic 5. Absorbance of the different membranes washed outside the holder, and inside the holder at different flow rates.

5.3.3 Integration of the incubation of antibodies.

In this part of the project, all the stages were performed inside the holder (except for the washing stages) and the effectiveness was tested only with blank samples (no bacteria were filtered).

After the TMB incubation, all the membranes showed a saturated signal, as the solution turned deep green ***(Image 10)***. As the liquid was too colored, there was no possibility of extracting it and read the absorbance in the ELISA, as there was a risk of saturating the reader.

One of the possible explanations of the saturated signal could be that the methacrylate from the holder presents a higher affinity for antibodies than the membrane itself. By introducing the antibodies through the slot, instead of joining the membrane, they bind to the walls of the holder. Although, further work is needed to study the interaction between the methacrylate and the antibodies.

This evidence, added to the fact that the trials are performed one after another without washing the holder, could have given the error causing the accumulation of antibodies and subsequently the saturation of the TMB.

Image 10. Saturated membranes. Own source.

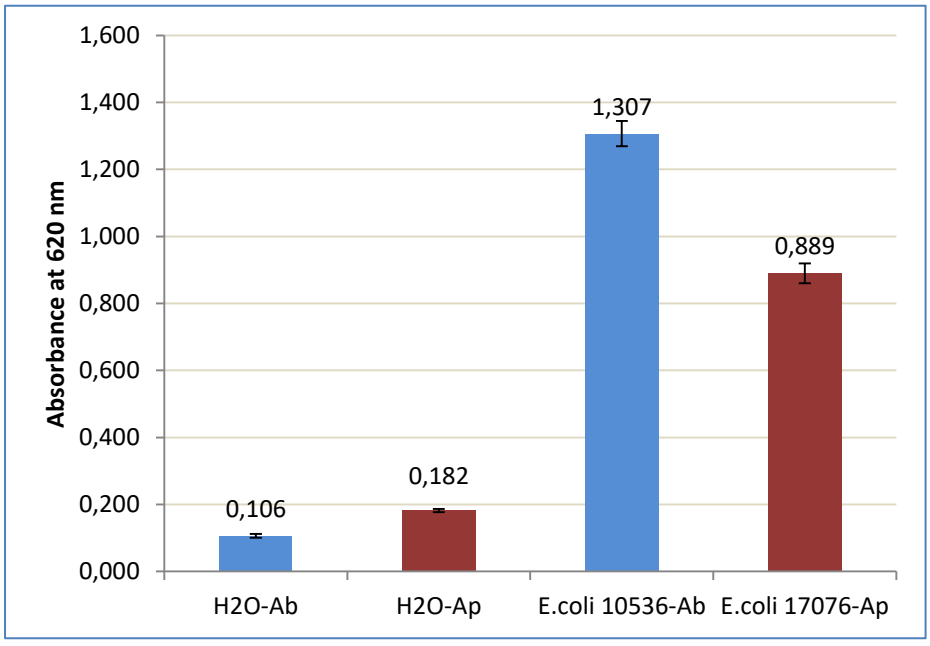
5.4 Perform a preliminary study with the aptamer.

The aim of this study was knowing if the aptamer is able to bind cells of *Escherichia coli* in polycarbonate membranes, following the same pattern as the antibody. The aptamer is a nucleic acid that presents advantages over the antibody as: temperature resistance and high specificity [15]. In this assay, we've used a specific aptamer that binds *Escherichia coli* 17076 (different from the antibody, 10536).

The protocol was performed in the same conditions, but replacing the antibody by the aptamer. Three blank samples were considered (100 ml of distillated water filtered) and three samples with a 10^6 concentration (in 100 ml of distillated water). In ***Graphic 6***, the results obtained with the aptamer (red) and antibody (blue) are shown. These two results are not comparable as they don't come from the same bacteria culture (strain 10536 antibody was used and the aptamer strain 17076), but is necessary a comparison between them.

As shown in the graph, the aptamers bind the bacteria present in the suspension, and therefore, the blank suspension and the bacteria suspension can be distinguished.

Although the differences between the blank and the 10^6 dilution is lower than with the antibody protocol, this can be improved by adapting the different phases of the protocol to suit the aptamer better.



Graphic 6. Absorbance of blank samples and 10^6 bacteria samples, with antibodies and aptamers

6. CONCLUSIONS

1. Reducing the time of the 1st blocking membrane treatment.

After the pertinent studies, it has been determined that the time of immersion of the membrane in the detergent doesn't significantly affect the unspecific binding between the antibodies and the membrane. Therefore, to minimize the time of the protocol, we've passed from blocking the membranes for two hours to blocking them for 5 minutes.

2. Adapt new conditions for the study of the whole filter. New calibration curve.

The protocol has been adapted to measure the exact amount of bacteria attached in the whole membrane. A new calibration curve has been performed and shows a good linearity (R=0.98). Originally, the results were approximated as only pieces of the filter were analyzed. The study of the whole membrane is introduced to the protocol, making the results more reliable.

3. Integration of the protocol inside the holder

3.1 Integration of the 2nd blocking membrane treatment.

The results suggest that although blocking inside the holder is valid for reducing the unspecific binding, performing the blocking stage outside the holder, shows a greater reduction. There results may suggest that the amount of detergent used affects de permeability of the membrane to the antibodies (outside a higher amount of detergent is used). Further work is needed to integrate this stage in the best conditions.

3.2 Integration of the washing stages.

After the tests, the conclusion is that washing the membranes within the holder at the same conditions doesn't work, as the antibodies weren't removed successfully. The holder seems to interfere in the washings stages, as all the other conditions were identic to the original protocol.

3.3 Integration of the antibodies incubation inside the holder

After performing the incubation inside and observing a saturated signal, the hypothesis that the antibodies can be retained by the methacrylate from the holder rather than by the membrane itself is likely. More research is needed to study this hypothesis.

4 Preliminary study of the aptamer

The aptamer was efficient in joining the *chosen Escherichia coli* strain. It was possible to differentiate between the blank sample, and the bacteria sample. However, the assays with the antibody showed to be more sensible. It would be advisable to investigate more deeply on the optimal conditions that the aptamer needs to bind efficiently.

7. RECOMMENDATIONS

To move forward in this study, some suggestions are collected in the following section:

1. Reduce the number of membrane treatments by investigating more detergents that can reduce the unspecific binding between the antibodies and the membrane with only one treatment.

2. Substitute the current colorimetric detection method with an amperometric sensor, as it turn out to be more sensitive, fast and the measure can be followed online.

3. Study different materials for manufacturing the holder or decreasing roughness of the methacrylate by polishing it, so the antibodies don't bind the holder.

4. Perform studies with a less specific aptamer that can detect all *Escherichia coli* strains, including 10536, so the results can be comparable to the antibody ones.

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9. ANNEX

Conditions		Absorbance			X	SD
Actual protocol	Blank	0,053	0,051	0,051	0,052	0,001
	Max. Union	2,967	2,967	2,967	2,967	0
	2h	0,066	0,064	0,063	0,064	0,001
	30 min	0,073	0,077	0,078	0,076	0,002
	15 min	0,069	0,066	0,067	0,067	0,001
	5 min	0,072	0,074	0,076	0,074	0,002
Previous protocol	Max. Union				1,862	0,137
	2h				0,029	0,03

Table 1. *Average absorbace an SD of the Reducing Blocking time assay*

Concentration	Absorbance			X	SD
Blank	0,112	0,101	0,111	0,108	0,006
10^3	0,397	0,525	0,569	0,497	0,089
10^4	0,936	0,786	0,796	0,839	0,084
10^5	1,074	1,176	0,928	1,052	0,175
10^6	1,254	1,307	1,295	1,280	0,037

Table 2. *Average absorbance an SD of the calibration curve.*

OUTSIDE					
Conditions	**Absorbance**			**X**	**SD**
Blank	0,121	0,094	0,079	0,105	0,022
10^4	0,514	0,709	0,503	0,575	0,116
10^6	1,262	1,182	1,321	1,255	0,070
INSIDE					
Blank	0,542	0,209	0,073	0,281	0,232
10^4	0,807	0,767	0,945	0,840	0,094
10^6	1,234	1,394	1,028	1,219	0,183

Table 3. *Average absorbance an SD of performing the 2nd blocking treatment, outside and inside the holder*

Flow rate	Absorbance			X	SD
0	0,34	0,359	0,268	0,322	0,048
0,25	0,487	0,485	0,637	0,536	0,087
0,5	0,341	0,232	0,351	0,308	0,066
1	0,344	0,231	0,252	0,276	0,060
1,25	1,347	0,988	1,17	1,079	0,129
1,5	1,334	1,253	1,373	1,320	0,061
1,75	1,993	2,353	2,163	2,170	0,180

Table 4. *Average absorbance an SD of performing the water washes at different flow rates*

Conditions	Absorbance			X	SD
Washing outside	0,156	0,175	0,145	0,159	0,015
0,5 ml/min (Inside)	1,451	1,436	1,447	1,445	0,008
1 ml/min (Inside)	1,322	1,351	1,365	1,346	0,022

Table 5. *Average absorbance an SD of performing the washing stages inside and outside the holder at different flow rates*

Conditions	Absorbance			X	SD
H_2O-Ab	0,112	0,101	0,111	0,106	0,006
H_2O-Ap	0,176	0,184	0,185	0,182	0,005
***E.coli* 10536-Ab**	1,250	1,312	1.295	1,307	0,037
***E.coli* 17076-Ap**	0,856	0,899	0,913	0,889	0,030

Table 6. *Average absorbance an SD of performing the protocol with antibodies and aptamers*