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Original article

Comparison between the recovery rate of three concentration protocols of water samples intended for analysis by Molecular Biology: Membrane filtration, filtration on gauze pad and centrifugation

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ABSTRACT

The presence of microorganisms (bacteria, protozoa, viruses, etc.) in water is a crucial indicator of its quality and safety. The detection of these microorganisms by conventional and classical techniques is widely used in water quality control laboratories; nevertheless these methods have limitations in terms of rapidity and precision of results. The use of Molecular Biology has been a great evolution in the techniques of water analysis. However, the choice of the concentration protocol allowing for the best rate of microorganism recovery in a suspension remains a real challenge. The objective of this experimental study is to compare the recovery rate of three different protocols of water concentration (membrane filtration, filtration on gauze pad and centrifugation) for samples intended for analysis by polymerase chain reaction PCR. Which can then serve as a reference protocol for water quality control laboratories. The experimental results have shown that the membrane filtration protocol yields the best recovery rate and concentration of microorganisms followed by filtration on gauze pad, while the centrifugation protocol (8000*g*, 10 min, 22 °C) gives the lowest rate of recovery out of the three protocols. The experimental results obtained through this study allows us to contribute to the optimization and standardization of water samples concentration techniques intended for analysis by Molecular Biology.

1. Introduction

Water is a crucial element for the survival of all living beings; however different sources of water pollution (microbiological, chemical, etc.) can damage human and animal health and disrupts the integrity of the environment (Owa, 2013).

All forms of water are susceptible to be affected by microbial contamination: surface water, groundwater, sea water, and even ice. Contamination comes from various ways, a large part of which is linked to human activities: disposal of untreated wastewater, reuse of insufficiently treated effluents, and use of animal waste as manure (Pinon and Vialette, 2018).

Many infectious pathogens excreted by infected hosts (human or animal) can be transmitted by water to new hosts (Medema, 2013). These pathogens can cause several diseases, known as water-borne diseases such as gastroenteritis, cholera, typhoid, amebiasis (Peterson, 2001) These Infectious diseases are commonly transmitted by direct or indirect contact (Nwabor et al., 2016). These diseases are considered as the main causes of human morbidity and mortality in the world (Griffiths, 2008) and sometimes may lead to epidemics (Majdoub et al., 2004).

The risk of emergence of water-borne diseases increases where sanitation and personal hygiene standards are insufficient (Rusinol and Girones, 2017).

Escherichia coli and enterococci are the traditional indicators of water fecal contamination and its provide an assessment of the potential presence of other human pathogens (Botes et al., 2013).

The presence of microorganisms in the water remains an important indicator of the health of the populations and environment.

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Classical and conventional methods of analysis are frequently used in laboratories carrying out the control and monitoring of water quality. However, these methods are excessively time-consuming (presumptive, confirmatory test) and in some cases some microorganisms such as viruses may be difficult to detect or they are not in a sufficient quantity in the water samples to be able to be detected. The use of the quantitative polymerase chain reaction (qPCR) method constitutes an alternative to culture-based microbiological methods for the detection and quantification of microorganisms (Bouchez et al., 2017) and presents an effective tool to detect and quantify microorganisms within water in a few hours (Botes et al., 2013).

Contrary to the majority of biological samples for which PCR analysis involves extraction followed by amplification and detection, water samples imperatively require an initial phase of sample concentration. Microorganisms (bacteria, protozoa, viruses, etc.) are found dispersed in water matrices, the presence of suspended matter and other elements presents a difficulty at the time of analysis, hence the need to go through a phase of separation and concentration before proceeding to their research and detection.

There are several concentration protocols (e.g membrane filtration, filtration on gauze pad, centrifugation, etc.).The choice of a suitable one that can yield satisfying recovery rates is a real difficulty for analysts. Centrifugations and filtration with its different supports (membrane, gauze pad ...) are the usual and commons particle separation methods used in different fields of microbiology.

Centrifugation involves the application of centrifugal force (Stephenson, 2016). It is a protocol used to separate particles in a solution based on their size, shape, density, medium viscosity and rotor speed. The main advantage of this technique is that, it's a simple protocol that allows the isolation of more than two types of cells; however centrifugation is limited to small volumes of water (World Health Organization, 2003). Moreover, the low purity of this technique can be harmful to cells of centrifuged microorganisms.

Membrane filtration has many benefits such as: It is a simple and fast protocol adapted to any volumes of non-turbid water. The size and the structure of the filtration medium can be a factor in the selection of the microorganisms to be tested. It is not expensive and it can be used with various types of membranes (with different composition and porosity) according to the microorganisms tested. However, the major disadvantage of this technique is the risk of membrane clogging; therefore, turbid water cannot be filtered .Also, this protocol requires a high differential pressure to function

Filtration on gauze pad has the same benefits as membrane filtration, in addition this technique can be used as a measure for turbid water (sewage and wastewaters) for which they were originally developed (Wyn-Jones and Sellwood, 2001) also filtration on gauze pad performance can be improved by the addition of an adjuvant (ISO19250, 2010).

This research paper presents an experimental study that provides support to laboratories analysts operating in the water analysis sector in order to choose the best concentration protocol allowing for better recovery of microorganisms and subsequently serving as a reference protocol.

2. Materials and methods

The methods based on molecular biology for water samples analysis require necessarily an initial phase of concentration. Several techniques have been described (Le Guyader et al., 2014). The choice of a technique among them that offers a better recovery rate of microorganisms is important. This experimental study compares three concentration protocols of water samples intended for analysis by Molecular Biology. The first approach is called membrane filtration using a membrane with 0.45 μm pore size and 47 mm of diameter (Haugland et al., 2005; Delarras, 2014). The second is filtration on gauze pad (Manor et al., 1999; Wyn-Jones and Sellwood,2001; ISO19250, 2010; Sikorski and Levine, 2020) and the last one is centrifugation protocol using 8000*g*, 10 min, 22 °C (Aw et al., 2012; Maal et al., 2015; Lall et al., 2016; Fu et al., 2020).

Two matrices were chosen to perform this experimental work: 1) natural water matrix (surface water) with a low concentration of microorganisms and suspended matter and 2) a wastewater matrix characterized by a high concentration of microorganisms and suspended matter. The objective is to ensure that the results obtained are independent from the type of matrix and test the effectiveness of the concentration protocols in the cases of samples rich or poor in suspended matter.

In order to compare between the recovery rates of different concentration protocols and to be sure to obtain positive results that they can be compared subsequently, the two waters samples (natural and wastewater) used in this study were doped with a determined concentration of a strain of *E.coli* and then were subdivided into three parts each of them underwent the three different concentration protocols discussed above followed by a common extraction phase.

The extraction of nucleic acids can be executed through chemical lysis (guanidium thiocyanate), enzymatic lysis (proteinase K), also other protocols using temperature (boiling) can be used (Le Guyader et al., 2014). In this study we used magnetic beads technology to extract the *E. coli* DNA. Finally, the amplification and detection have been achieved by real-time PCR, the results obtained were compared in order to determine the concentration protocol providing the best recovery rate. In what follows, a detailed explanation of the experimental protocol used in this study is presented.

2.1. Presentation of concentration protocols used in the experiment

2.1.1. Filtration

Filtration is a separation process allowing the concentration of suspended species on a support, generally a sterile filter membrane (cellulose nitrate, cellulose ester, etc.), with a porosity of 0.2 or 0.45 μm depending on the size of the microorganism analyzed and generally 47 mm in diameter (Haugland et al., 2005; Delarras, 2014), or using a sterile gauze pad(Manor et al., 1999; Wyn-Jones and Sellwood, 2001; Sikorski and Levine, 2020) also a sterile adjuvant of filtration can also be added (ISO19250, 2010).

2.1.2. Sonication

Sonication is a process in which sound waves are used to agitate solution containing particles. It is usually applied using an ultrasonic bath or an ultrasonic probe. Sonicators either produce sound waves in a water bath, where samples are placed, or they can be in the form of probes directly attached to the sample to be sonicated (Taylor, 2010;Chung, 2017; Kamineni and Huang, 2019;Thanu et al., 2019).

2.1.3. Centrifugation

Centrifugation is one of the most employed techniques in the molecular biology laboratory (Stephenson, 2016). It's based on the separation of particles according to their size and density (Zhou, 2012). There is several types of centrifuge depending on their rotors. Different types of rotors are used for different applications. For example, the most frequently used in an environmental water samples laboratory is the fixed-angle rotor due to their ability to pellet. Bacterial cells can usually be pelleted with a

centrifugation speed of 8000*g*, 10 min (Aw et al., 2012;Maal et al. 2015; Lall et al. 2016; Fu et al., 2020).

2.2. Sampling

Samples were collected from two different types of water matrices: surface natural water (river) and waste water (raw waste water before treatment). Sterile bottles with a volume of 500 ml were used for taking samples.

2.3. Determination of the initial concentration of samples in Escherichia coli

In order to determine the initial load of *E. coli* in the samples, an initial analysis was applied using the most probable number method according to NM ISO 9308–2 method, this technique is based on the growth of target organisms in a liquid medium, incubation and calculation of the "Most Probable Number" (MPN) of organisms according to MPN tables (NM ISO 9308-2, 2019).

Table 1 represents the results of *E. coli* enumeration in water samples.

2.4. Doping with a determined concentration of Escherichia coli

Before proceeding to the concentration step, the samples were doped with a determined concentration of a bacterial strain of *Escherichia coli* ATCC 25922. The strain of *E. coli* stored at −80 °C was thawed and then the biochemical profile of this strain was confirmed using biochemical identification system API 20E and Gram stain. This strain was then cultured in a nutrient broth (TSB) incubated at 37 °C for 21 ± 3 h; from this broth, a dilution series was prepared in order to determine the initial concentration of this strain and for each dilution we proceeded to the incorporation of 1 ml using nutrient agar type Plate Count Agar (PCA). Table 2 represents the required results of the incorporation.

The initial concentration of *Escherichia coli* strain can be estimated at 79.10^7 CFU (Colony Forming Unit). To determine the recovery rate of the concentration protocols a volume of one milliliter of the dilution 10^{-7} was added to the bottles containing 500 ml of water sample (natural and waste waters). After the sample had been well homogenized and distributed in three sterile bottles of 100 ml (three bottles per matrix) each one was used for a concentration protocol (membrane filtration, filtration on gauze pad, centrifugation).

2.5. Concentration of samples

Three types of concentration protocols were tested for the same initial concentration of *E. coli*.

2.5.1. Membrane filtration

100 ml of each sample (natural water and waste water) doped with the *E. coli* was filtered through Millipore type cellulose nitrate membranes with porosity of 0.45 μm and diameter of 47 mm. The membrane was then introduced into a bottle containing 10 ml of ultrapure sterile water. This bottle subsequently underwent a sonication phase using an ultrasonic bath for 10 min in order to detach and recover the bacteria stuck to the filter, the membrane was then eliminated while the volume of the concentrate (10 ml) was subsequently used for the extraction phase.

Table 1
Ecoli enumeration results.

	Results (MPN/100 ml)
Waste water	$5.\ 10^4$
Naturel water	3.10^2

2.5.2. Filtration on gauze pad

Gauze pads were prepared and sterilized at 121 ± 3 °C for 15 min and used for the filtration phase. Before proceeding to the filtration, 1.5 ml of the diatomaceous earth solution (adjuvant of filtration) (ISO19250, 2010) was introduced into 100 ml of the samples (natural and wastewaters), doped with the strain of *E. coli*. A volume of 15 ml of diatomaceous earth was also filtered first through the gauze pad to form a first layer, before filtering the 100 ml of sample. Subsequently the gauze pad was placed in a sterile bottle containing 10 ml of ultrapure sterile water, this bottle underwent afterwards a sonication phase using an ultrasonic bath for 10 min, in order to detach and recover the bacteria stuck to the gauze pads.

The gauze pads were then eliminated while the volume of the concentrate (10 ml) was subsequently used for the extraction phase.

2.5.3. Centrifugation

Samples were transferred to tubes of 100 ml to be centrifuged at 8000*g*, 22 °C for 10 min. The supernatant was discarded and the pellet was resuspended with ultrapure sterile water in order to obtain a final volume of 10 ml in each tube.

2.6. Extraction of nucleic acid

The nucleic acid was extracted and purified from each concentrated sample using a kit based on magnetic beads. Thus, the samples are mixed using zircon beads in a solution based on guanidinium thiocyanate. The extraction and purification were performed using an automaton.

2.7. Amplification and detection

The step of amplification and detection has been carried out by the Applied Biosystems 7500 Fast RT-PCR Thermal Cycler**.**

2.7.1. Reaction mixture

According to the kit used to detect the nucleic acid of *E. coli*, the reaction mixture is prepared as shown in the table below (see Table 3).

The internal positive control (IPC) included in the kit is used to monitor PCR inhibition. Considering that this kit does not contain a positive control, we used the nucleic acid extracted from the cultured strain of *E. coli* ATCC 25922 as positive control.

2.7.2. Thermal cycling conditions

Thermal cycling conditions for the 7500 Fast Real-Time PCR Instrument have been programmed according to the following table (see Table 4).

3. Results

The comparison between the recovery rates of these three concentration protocols used to concentrate water samples intended for analysis by molecular Biology Analysis (PCR) was carried out through the amplification curves and cycle threshold (Ct) values obtained by PCR.

The concept of the "threshold cycle" is the basis of an accurate and reproducible quantification for techniques fluorescent in PCR (Poitras and Houde, 2002). The cycle threshold (Ct) can be defined as the thermal cycle number at which the fluorescent signal exceeds that of the background and thus passes the threshold for

Table 2
Results of incorporation of *E coli.*

Bacterial concentration	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Incorporation results	>300	>300	>300	>300	>300	>300	79	8

Table 3
Preparation of the reaction mixture.

Component	Sample type	
	Test sample	Negative control
10× Assay Mix	3 µl	3 µl
2× Environmental Master Mix 2.0	15 µl	15 µl
Sample DNA	Up to 12 µl	–
Nuclease –free water	To 30 µl total	12 µl

Table 4
Thermal cycling conditions.

Stage	Stage 1	Stage 2	
Steps	Enzyme activation	Denature	Anneal/extend
Number of cycle	1 cycle	45 cycles	
Temperature	95 °C	95 °C	60 °C
Time	10 min	15 sec	45 sec

positivity. Ct value can be directly correlated to the starting target concentration for the sample (Stratagene, 2004).

In a real time PCR assay, a positive reaction is detected by the accumulation of a fluorescent signal. The Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold. Ct levels are inversely proportional to the amount of target nucleic acid in the sample. The lower the Ct value the higher the quantity of genetic material in the sample. Ct values obtained in this way are semi-quantitative and are able to distinguish between high and low bacterial loads of *E. coli*.

The results obtained from this experimental study show clearly that the three concentration techniques tested allowed the detection of the bacterial DNA of the *E. coli* target for both natural water and wastewater.

The highest Ct values were recorded for the centrifugation technique; followed by gauze filtration where the ct values of the membrane filtration are the lowest. The same conclusion was obtained for the natural water matrix as well as for the waste water. The difference in the Ct values provides direct information on the difference in the initial load of *E. coli* strain and therefore on the recovery of each concentration protocol. The same conclusion was obtained for the natural water matrix as well as for the waste water. The difference in the Ct values provides direct information on the difference in the initial load of *E. coli* strain and therefore on the recovery of each concentration protocol.

In order to ensure the quality of the results produced, the detection kit used also includes an internal positive control (IPC), consisting of reference genes that will be amplified together with the target sequence during the same PCR reaction run (Moldovan and Moldovan, 2020).

The objective is to ensure that DNA was appropriately extracted from the sample and to detect the presence of extremely negative results (when the pathogen tested shows amplification but there is no internal control DNA amplification) or the presence of inhibitory substances in the sample (Paxson, 2008).

All the internal positive controls showed a positive amplification, which confirms that, the extraction and amplification steps were successful and inhibitory substances were absents. In addition, the results of the positive and negative controls that accompanied the cycle were conforms. Which allows the validation of the results obtained.

Table 5 shows the Ct values according to the concentration protocol used and to the type of matrix

Fig. 1 represents the amplification curves obtained by PCR for the three concentration protocols and for the two matrices tested.

The amplification curve in the Fig. 1 is represented by the evolution of Delta Rn versus cycle number. Deltas Rn correspond to the magnitude of the fluorescent signal generated by the reporter at each cycle during the PCR amplification. It is the difference between Rn value of an experimental reaction and the Rn value of the baseline signal generated by the instrument (Lockey et al., 1998).

Fig. 2 shows the representation of the Ct values for each concentration technique. We can clearly remark that for both types of water, centrifugation gives the greatest values of Ct followed by the technique of filtration on gauze while filtration on membrane gives the lowest values of Ct.

4. Discussion

The results obtained after amplification and detection by PCR shows:

The presence and detection of *E. coli* in all doped samples: natural water and waste water.

The Ct values obtained are different and depend on the concentration protocol: membrane filtration, filtration on gauze pad or centrifugation.

The samples that were concentrated by the centrifugation protocol (8000*g*, 10 min, 22 °C) show the highest Ct values.

Samples that were concentrated by the membrane filtration technique or by filtration on gauze pad have comparable Ct values and are always lower than the values obtained by the centrifugation technique for the same types of samples.

The highest Ct values are those of the centrifugation protocol followed by filtration on gauze pad while those of the membrane filtration are the lowest.

Since Ct values are inversely proportional to the initial concentration of microorganisms it can be concluded that the concentration protocol based on membrane filtration allows for best recovery rate of microorganisms followed by the gauze filtration protocol and finally the centrifugation protocol (8000*g*, 10 min, 22 °C). Furthermore, the results of the positive and negative controls and IPC were compliant which allows validating the PCR plate.

Others experimental studies based only on filtration protocol for bacterial concentration (*E. coli*) in water sample (Shrestha and Dorevitch, 2019) or that combine the protocol of filtration - sonication followed by a centrifugation for bacteria concentration

Table 5
Ct values of *E.coli* amplification according to the concentration protocol.

Protocols of concentration	Ct values	
	Naturel water	Wastewater
Cenrifugation	27.40	29.25
Filtration on gauze pad	20.36	21.23
Membrane filtration	19.49	20.19

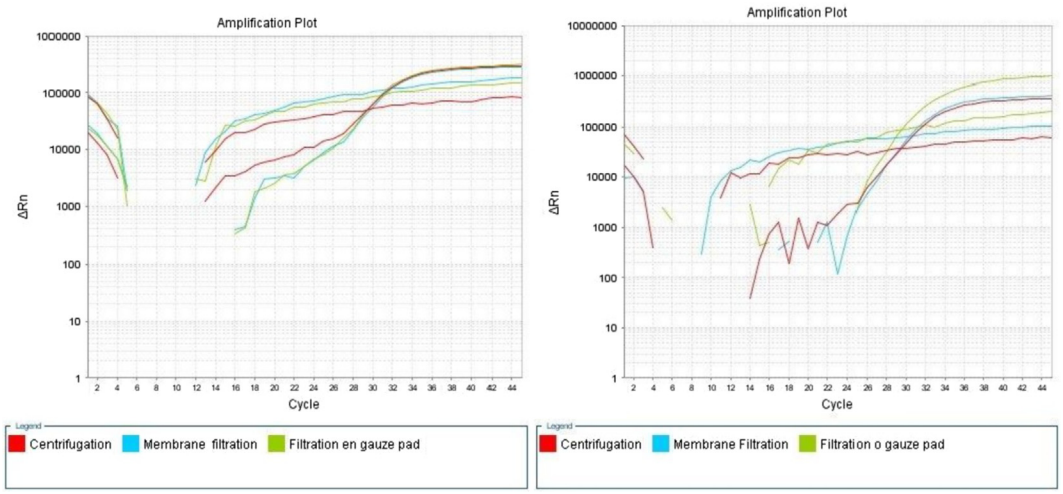

Fig. 1. Result of amplification using three different protocol of concentration natural water sample (left); waste water sample (right).

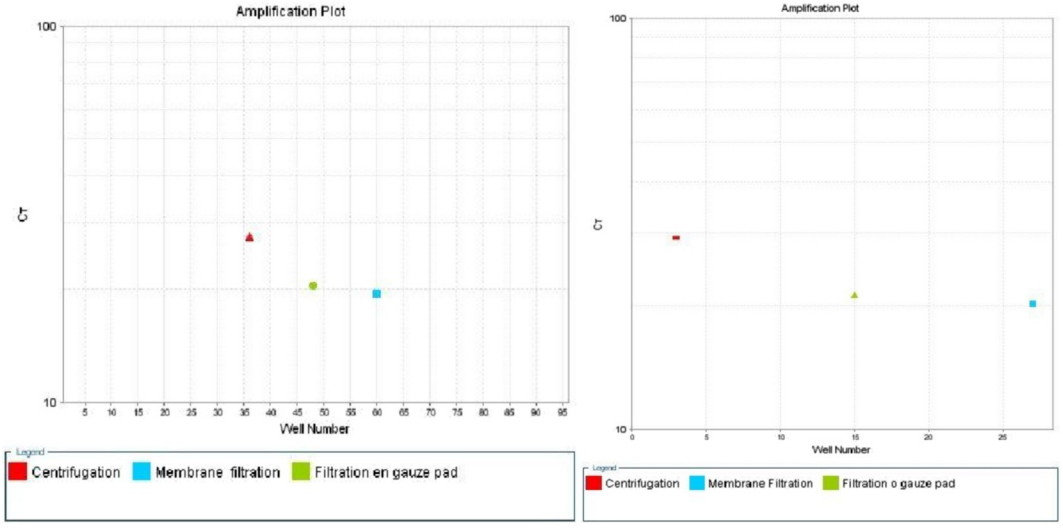

Fig. 2. Result of Ct values using three different protocol of concentration natural water sample (left); waste water sample (right).

(*Legionella pneumophila* and *E. coli*) (Wolf-Baca and Siedlecka, 2019) allowed the detection of bacterial cells of the tested species.

Also and in order to provide a solution to the problem related to bacterial viability detected by molecular biology techniques, an experimental study combining filtration of water samples and pre-enrichment in a non-selective (Delabre et al., 1998) or selective (Isfahani et al., 2017) medium followed by purification and detection of DNA confirmed the detection of the bacteria tested.

Traditional methods of microorganisms detection in water samples have limitations (low specificity and accuracy, long incubation period, etc.) and are unable to cover all parameters. Detecting these microorganisms with molecular techniques is highly suggested as a new approach allowing very specific and rapid detection. The concentration phase of samples constitutes a decisive step. The comparison between the recovery rates of three concentration protocols in this experimental study has clearly shown that the concentration protocol based on membrane filtration allows for the best recovery rate of microorganisms followed by the gauze filtration protocol and finally the centrifugation protocol (8000*g*, 10 min, and 22 °C).

The centrifugation is considered a robust technique but the loss of bacterial biomass by the concentration protocol of water using centrifugation compared to the filtration techniques, can be explained by different factors such as: the choice of centrifugation speed and duration, the technique used to discard the supernatant (siphoning, . . .) and the alteration of bacterial cell surface properties and internal structures, including DNA due to the centrifugal compaction (Peterson et al., 2012).

Other experimental works were carried out in order to compare different water concentration protocols. For example, Villari et al. (1998) compared two protocols of concentration of Seeded water samples (40 ml) in different conditions: Filtration (0.2 μm pore-size polycarbonate or mixed esters filters) and centrifugation (8150 g for 15 min or 3800*g* for 30 min). The results of their study have shown that the recovery of the filtration is generally superior to centrifugation. Also, the results of experiments carried out by Thomson et al. (2008) confirm that filtration technique (using 0.45-m cellulose nitrate filters)for the isolation of mycobacteria from water samples is a more sensitive method for concentration than centrifugation (5,000*g* for 20 min at 25 °C).

So as to consolidate the results obtained, additional tests using other types of bacteria for doping and using other matrices (treated water, beach water) are well recommended and in order to evaluate the losses linked to the performance of the centrifugation

protocol, other experimental studies including other conditions of centrifugation (speed, time, etc.) can be addressed.

5. Conclusion

From this experimental study, it was concluded that for the same samples (naturel water or waste water) doped with the same concentration of *E.coli* strain having undergone three different concentration protocols, namely (membrane filtration, filtration on gauze pad and centrifugation) while the extraction and detection steps by PCR were similar, the Ct values were different. Also, these values were always higher for the centrifugation protocol followed by filtration on gauze and finally membrane filtration for all of the types of matrices tested (natural water, waste water).

This study allows us to deduce that the protocol of membrane filtration gives the best rate of recovery and concentration of microorganisms followed by filtration on gauze pad, inversely the centrifugation protocol (8000*g*, 10 min, 22 °C) gives a low concentration rate compared to the other two protocols.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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