A proof-of-concept of a passive sampler for microorganism sampling, with potential for patenting the developed methodology

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4	BioDetection Systems B.V.	BDS
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6	German Environment Agency	UBA
7	Helmholtz-Centre for Environmental Research	UFZ
8	Consorci Concessionari d'Aigües per als Ajuntaments i Industries de Tarragona	CAT
9	Tutech Innovation GmbH	TUTECH
10	Metropolitana Milanese SPA	MM
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12	The National University of Water and Environmental Engineering	NUWEE

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Abstract

Current active sampling methods, such as grab or composite sampling, are not suitable to monitor and detect sporadic events such as pathogenic contaminations of drinking water distribution networks (DWDN). Using grab sampling, the probability of detection is too low, while composite sampling might require handling huge volumes of water. Concerning online sampling, even if automatic monitoring could increase the chances of detection of biological instability of water in the supply system, the costs could be prohibitive. In this framework, a passive sampler for microorganisms could be an effective and cheap early-warning system to promptly identify contamination events. However, this methodology is not enough developed. Hence, the goals of this work were: (i) to develop a standardized experimental protocol to test the suitability of materials to behave as passive samplers for bacteria detection; (ii) to validate the developed protocol, evaluating the performances of some materials, both commercial and specifically developed, to have a proof-of-concept of the sampling methodology, to be exploited in a DWDN early warning system by water utilities.

In detail, two experimental protocols were developed, based on the contact conditions between bacteria and passive sampler: the first one, relying on batch experiments, is devoted to the screening of the best material to be used; the second one, relying on in-flow contact conditions, is aimed at validating the suitability of the selected material in "environmental" conditions, meaning drinking water. Concerning the proof-of-concept of the passive sampling, an in-flow cell was installed in the Feltre DWTP (Milan, Italy) to assess the performance of the passive sampler when immersed in drinking water for longer time (1 d, 3 d and 7 d). Experiments were performed exploring many conditions for generalization purposes: (i) different materials, being commercial materials (cellulose gauze) and specifically designed and produced materials (PAN-EDA membrane and cellulose nanosponges); (ii) several microorganisms having different characteristics (*Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis* and total heterotrophic bacteria (THB)); (iii) two water temperatures (12°C and 25°C) considering both groundwater and surface water as source water feeding the drinking water supply systems; (iv) several enumeration methods targeting different microorganisms' features (ability to grow in plate, integrity of the cells, DNA).

Based on the positive results obtained, this work made possible a step ahead in the development of a reliable sampling methodology targeted on bacteria, that could be valuable for water utilities. Actually, passive sampling would provide a cheap and effective monitoring system for promptly detecting sporadic events, such as the occurrence of pathogens in the DWDN.

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Abbreviations

bPEI	Branched Polyethylene Imine
CFU	Colony Forming Units
CNS	Cellulose-based Nanosponges
CT	Threshold Cycle
DWDN	Drinking Water Distribution Network
DWTP	Drinking Water Treatment Plant
EDA	Ethylene Diamine
GC	Genomic Copies
gDNA	Template DNA
IEP	Isoelectric Point
NOM	Natural Organic Matter
PAN	Polyacrylonitrile
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
qPCR	Quantitative Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
THB	Total Heterotrophic Bacteria count at 36°C
TOCNF	Thermally Cross-linking Oxidized Cellulose Nanofibers
TSA	Tryptic Soy Agar

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1. Introduction

Passive sampling is a method based on immersing a collecting medium, i.e. the passive sampler, in a bulk medium, i.e. water. During the immersion, a spontaneous exchange of microorganisms occurs between the two (Bivins et al., 2022). The main advantages of this approach are that the passive samplers are usually cheap devices, and they can be deployed for long monitoring periods (i.e., hours or days). Moreover, they are practical for sampling in remote areas and there is no need of pumping systems or power supply (Salim & Górecki, 2019). Compared to grab sampling, passive sampling might increase the chances of identifying sporadic events, such as the presence of pathogens in the DWDN. In turn, compared to composite sampling and online sampling, these approaches have the limit of being more logistically demanding or expensive than passive sampling.

In this project, our goal was to develop a proof-of-concept of passive sampler for microorganisms sampling.

This work provided several elements of innovation. Indeed, passive sampling is already a well-established method for chemical (organic) compounds, in both water and air (Górecki & Namieśnik, 2002). On the other hand, regarding microorganisms, there are still no standardized passive samplers for these types of pollutants. Indeed, while recent research focused on SARS-CoV-2 detection (Jones et al., 2022; Hayes et al., 2022; Hayes et al., 2021; Liu et al., 2022), studies on passive samplers for bacteria are rare. In addition, while most researchers focused on wastewater (Hayes & Gagnon, 2024), there is a lack of studies that targeted drinking water. Lastly, a standardized experimental protocol is lacking to assess quantitatively the suitability of materials to act as passive samplers, namely their ability in trapping and releasing bacteria for enumeration. Summarizing, the elements of innovation regard: (i) the type of targeted pollutants (bacteria); (ii) the type of targeted water matrix (drinking water); (iii) the development of a standardized experimental protocol relying on both batch and in-flow tests.

2. Set-up and procedures

In this section, the developed experimental protocols and their set-up are described. The first protocol refers to batch experiments, aimed at assessing the suitability of a material as passive sampler. The second protocol refers to in-flow experiments as validation of the ability of a material to behave as passive sampler. Both protocols comprise a contact step and a detachment step. During the contact step, bacteria can attach on the material used as passive sampler. During the detachment step, attached bacteria are forced to detach into a PBS solution, then used for microorganisms' enumeration.

2.1 Batch experiments

A scheme of the experimental set-up is shown in Figure 1. As for the contact step, it involves four Erlenmeyer flasks (250 mL), called *F1, F2, F3, F4,* and one flask for the detachment step, *F5*. The *F1* flask contains the sterilized PBS solution, acting as blank control. The flask *F2* contains the sterilized PBS solution and the passive sampler to be tested, acting as blank control for potential contamination deriving from the material. The flasks *F3* and *F4* contain the sterilized PBS solution spiked with the bacterial stock solution to obtain a concentration of 2.5 CFU/mL of the target bacteria. In flask *F4,* the passive sampler is also placed. Regarding the detachment step, the passive sampler, after the contact time, is removed from flask *F4* and placed in flask *F5*, filled with 125 mL of the sterilized PBS solution, to be used for detachment. During the contact step, lasting 4 h, the flasks are continuously mixed. After the contact time, the test solutions are sampled for bacteria enumeration. As for the detachment

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step, the flask *F5* is mixed for 20 s and then sonicated at 40 kHz for 2 minutes. After this, the test solution is sampled and used for bacteria enumeration.

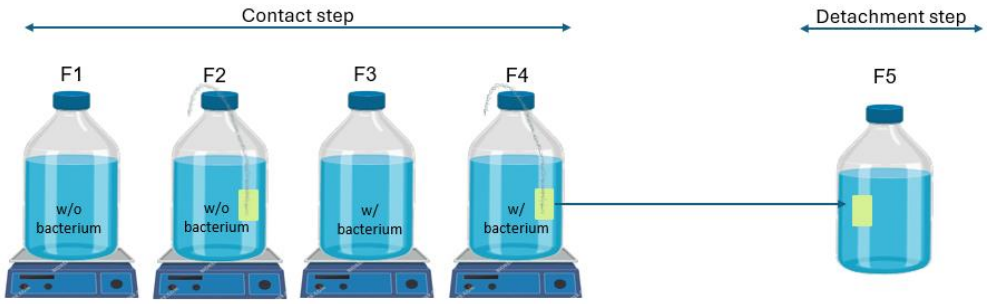

Figure 1. *Experimental set-up for batch experiments.*

The performance of the passive sampler is expressed in terms of two parameters, called Contact efficiency (η_C) and Detachment efficiency (η_D):

$$\eta_C = \frac{N_{F3} - N_{F4}}{N_{F3}} * 100 \quad (1)$$

$$\eta_D = \frac{N_{F5}}{N_{F3} - N_{F4}} * 100 \quad (2)$$

where N_{F3} and N_{F4} are the target bacteria concentrations after 4 hours mixing in flasks *F3* and *F4* respectively. N_{F5} is the target bacteria concentration in flask *F5* after sonication. The unit of measure depends on the analytical method adopted for bacterial enumeration and it is specified in section 4.

2.2 In-flow experiment

The in-flow experimental protocol relies on the experimental set-up shown in Figure 2. In detail, the passive sampling in-flow cell, specifically designed and built for this purpose, has been installed at the Feltre DWTP (Milan, Italy) at the outlet of the activated carbon filters, just before the inlet to the disinfection section. In Figure 2a, the scheme of the sampling system is reported, pointing out the sampling points and the type of samples to be collected: (i) grab sample at the inlet of the in-flow cell; (ii) grab samples at the inlet of the in-flow cell by the online analyzer Bactosense; (iii) passive samplers placed inside the in-flow cell. In Figure 2b each sampling point inside the Feltre DWTP is reported. The water volume passing through the passive sampling in-flow cell is measured by a water meter. The in-flow cell contains 9 passive sampler prototypes (Figure 2c and Figure 2d), immersed using steel bars, where they are tied using a fishing line.

The protocol contemplates the collection of a grab sample and of 3 out of 9 passive sampler prototypes after 1 d, 3 d and 7 d of immersion. About the samples automatically sampled by Bactosense, the analysis frequency is set on 6 h for a 7-day duration. Concerning the passive sampler prototype, each one is placed inside a sterilized Erlenmeyer flask, containing 125 mL of a sterilized PBS solution, as in flask *F5* in the batch experimental protocol. The flasks and the grab sample are transported to the laboratory inside a container with ice and immediately processed. Before analysis, the three flasks are sonicated for 2 minutes, and the sonicated solutions in each flask are then mixed.

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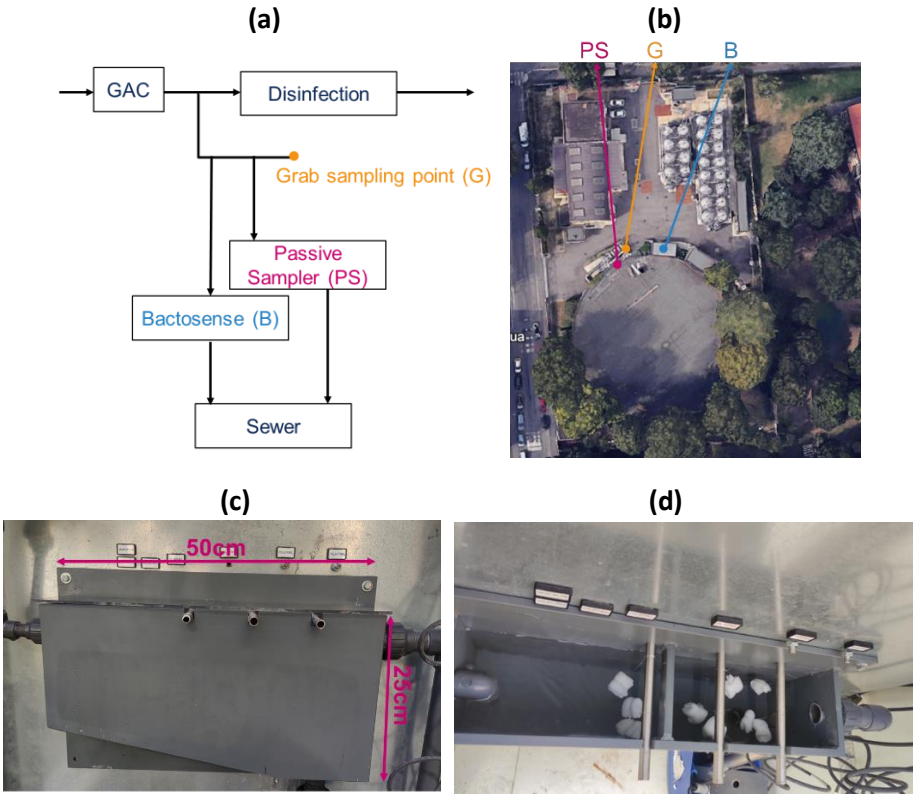

Figure 2. *In-flow experimental set-up: (a) scheme of the sampling system; (b) position of the sampling points in the Feltre DWTP; (c) passive sampling in-flow cell; (d) passive sampler prototypes inside the in-flow cell.*

3. Materials used as passive sampler

A passive sampler can be made of various materials which should promote both bacterial attachment on their surface and detachment for enumeration. In this project, three types of material were tested: (i) a commercial material, namely cellulose gauze; (ii) cellulose nanosponges (CNSs) developed by POLIMI; (iii) a porous polymer membrane (PAN-EDA) developed in cooperation with DVGW-TUHH.

In the following sections, each material is described.

3.1 Cellulose gauze

A picture of the cellulose gauze, used for both batch and in-flow experiments, is shown in Figure 3. This is a commercial product (PiC Solution, Stericompress Soft, Ultra gentle), having the following composition: 70% viscose and 30% polyester.

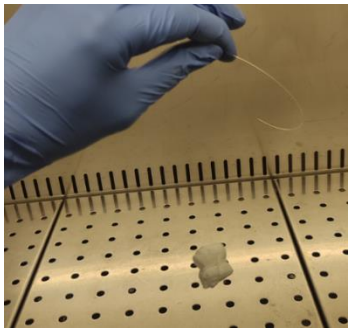

Figure 3. *Cellulose gauze tied with fishing line*.

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3.2 Cellulose nanosponges

CNSs are xerogels obtained by Thermally Cross-linking Oxidized Cellulose Nanofibers (TOCNF), bearing a variable amount of carboxylic groups on their backbone, and different polyamine polymers, presenting terminal primary amino groups. TOCNF-polyamine polymer water dispersions at variable w/w ratios promote the formation of hydrogels. These latter are put in molds and undergo freeze-drying followed by heating at about 100°C, as shown in Figure 4.

Figure 4. *CNS after freeze-drying (a) and heating (b).*

The thermal process promotes the formation of stable amide bonds between the two polymers. In some formulations citric acid (CA) is added in optimized amounts to trap the polyamine polymer, increase mechanical stability and try to prevent any potential polymer release.

Among polyamine polymers, we selected:

- Two bPEI derivatives, 25 kDa bPEI and 1.8 kDa bPEI, providing two CNS samples, SC1 (1 TOCNF: 1 bPEI 25kDa: 18% CA) and CNS2 (2 TOCNF: 1 bPEI 1.8 kDa).
- two branched PEG bearing terminal amino groups, PEG-4 arms and PEG-8 arms, providing two CNS samples, nL_SC3 (1 TOCNF: 2 PEG 4 arms) and nL_SC4 (1 TOCNF: 2 PEG 8 arms).

The selected polyamine polymers are shown in Figure 5.

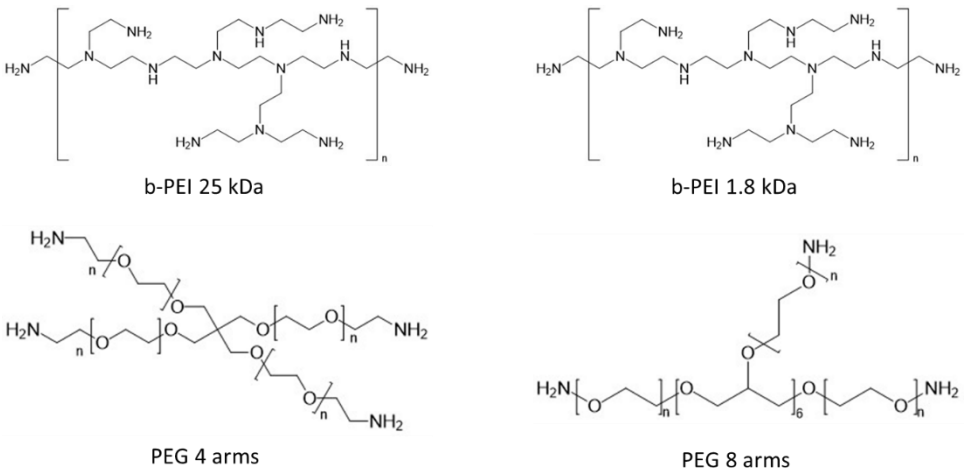

Figure 5. *Selected polyamine polymers for TOCNF cross-linking.*

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3.3 PAN-EDA membranes

Ethylene-diamine (EDA) modified membranes were tested as a passive sampler. The base material of the membrane is PAN, prepared as described in Scharnagl & Buschatz (2001) and subsequently modified according to the procedure outlined in Glass et al. (2021), to obtain the PAN-EDA membrane (see Figure a). This was provided by Helmholtz-Centrum Hereon.

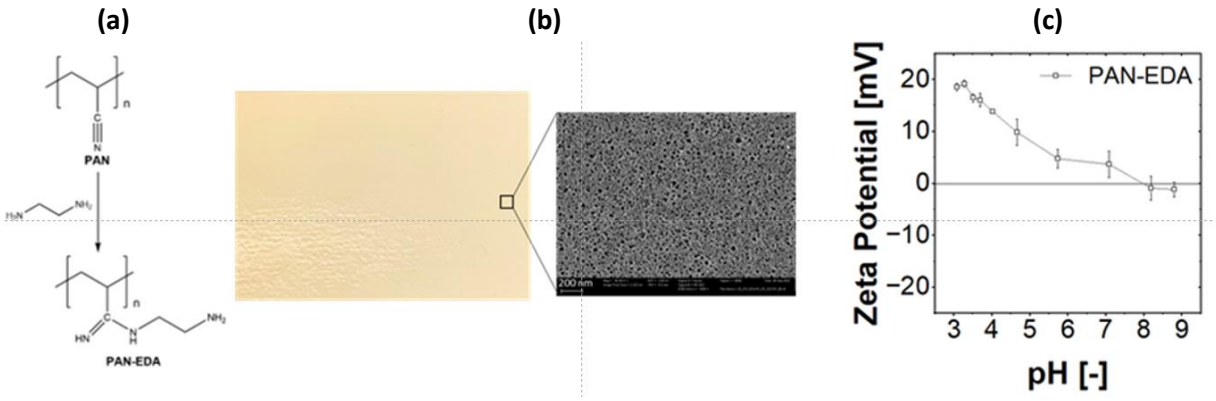

Figure 6. *(a) Schematic representation of the ethylenediamine modification of PAN membranes (PAN-EDA) prepared through a reaction with ethylenediamine (adapted from Glass et al. (2021)). (b) Picture of the PAN-EDA membrane and corresponding SEM image (adapted from Usman et al. (2024)). (c) Zeta potential of PAN-EDA membrane as a function of pH, showing an IEP at approximately pH 7.8 (adapted from (Usman et al., 2024)).*

The modification introduces EDA functional groups onto the PAN surface, incorporating the amine functionalities. The PAN-EDA membrane along with its corresponding SEM image is shown in Figure b, displaying a uniform and porous structure. As indicated by the zeta potential measurements (Figure c), the PAN-EDA membrane exhibited a positive surface charge below of approximately pH 7.8. This positive charge makes the membrane suitable for binding negatively charged species in aqueous environments. The positively charged surface has been demonstrated to effectively adsorb anionic contaminants, such as arsenate (Glass et al., 2021) and NOM (Usman et al., 2024), in water treatment applications. Given its promising adsorption properties, the PAN-EDA membrane was chosen to further investigate its potential as a passive sampler for pathogen detection.

4. Monitored microorganisms and enumeration methods

Different microorganisms and enumeration methods were used to develop and validate passive sampling. In detail, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and THB were tested in batch scale experiments.

The methods for bacterial enumeration used for both batch and in-flow experiments, were: (i) plate count, performed by POLIMI, MM, DVGW-TUHH; (ii) flow cytometry, performed by MM; (iii) quantitative PCR (qPCR) performed by DVGW-TUHH.

4.1 Plate count

The following analytical procedures were adopted for both batch and in-flow experiments: UNI EN ISO 9308-1:2017 for *Escherichia coli*; UNI EN ISO 7899-2:2003 for *Enterococcus faecalis*; UNI EN ISO 16266:2022 for *Pseudomonas aeruginosa*. THB were enumerated through membrane filtration and plating into TSA with incubation at 36°C for 24 h.

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4.2 Flow cytometry

Flow cytometry runs on the principles of light scattering, excitation, and emission of fluorescence. Fluorescently tagged cell components get excited when they pass through a laser beam, producing lights of different wavelengths. The fluorescence is used to count cells and analyze cellular properties. For routine quality control of water samples, flow cytometry tests offer a rapid alternative to conventional microbial plate counts. The reagents used for this analysis provide an easy-to-use and cost-effective test system to enumerate bacteria in many types of samples, spacing from industrial processed to drinking water.

Flow cytometry was used in some selected batch tests and online through Bactosense, discriminating between live and dead microorganisms, which was important to assess their physiological and metabolic state. The CyStain™ BacCount Viable kit was used to quantify the amount of live, damaged and dead cells.

4.3 qPCR

Quantification of bacterial 16S rDNA genes was performed using qPCR on a StepOne™ Real-Time PCR system (Applied Biosystems), employing SYBR Green PCR Master Mix (Qiagen). Each qPCR reaction of 20 µl consisted of 18 µl master mix (containing 2X SYBR Green Master mix, Applied Biosystems, Foster City, USA) and 2 µM of each respective forward and reverse primer) and 2 µl of template DNA.

For quantification, two universal primers, 926F (AACTCAAAGGAATTGACGG) (Lane, 1991) and 1062R (CTCACRRCACGAGCTGAC) (Allen et al., 2005), were used. The qPCR cycling conditions were as follows: an initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, primer annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 10 min.

Standards were prepared from purified PCR product using primers 533F/1492R, and these were diluted in a 10^0-10^8 serial dilution series after fluorometric concentration determination. Standards were stored at -20°C until use.

5. Applications and results

In this section, the main experimental results obtained by the adoption of the two developed protocols are summarized.

5.1 Batch experiments

The results obtained applying the batch experimental protocol are here described, grouped by the enumeration method adopted.

5.1.1 Enumeration by plate count

The results obtained by POLIMI and MM on *E. coli*, *Enterococcus faecalis*, *P. aeruginosa* and THB performing experiments at two different water temperatures (12°C and 25°C) are shown in Figure 7. *P. aeruginosa* displayed the highest contact efficiency, while THB the lowest one. Concerning the detachment efficiency, there are not relevant differences among the target bacteria. Comparing contact and detachment efficiencies at 12°C and 25°C, at the lowest temperature, the contact efficiency for *E. coli* and *Enterococcus faecalis* increase, while no difference was found for THB. In turn, detachment efficiency at 12°C decreases for *Enterococcus faecalis* and THB, while it remains constant for *E. coli*.

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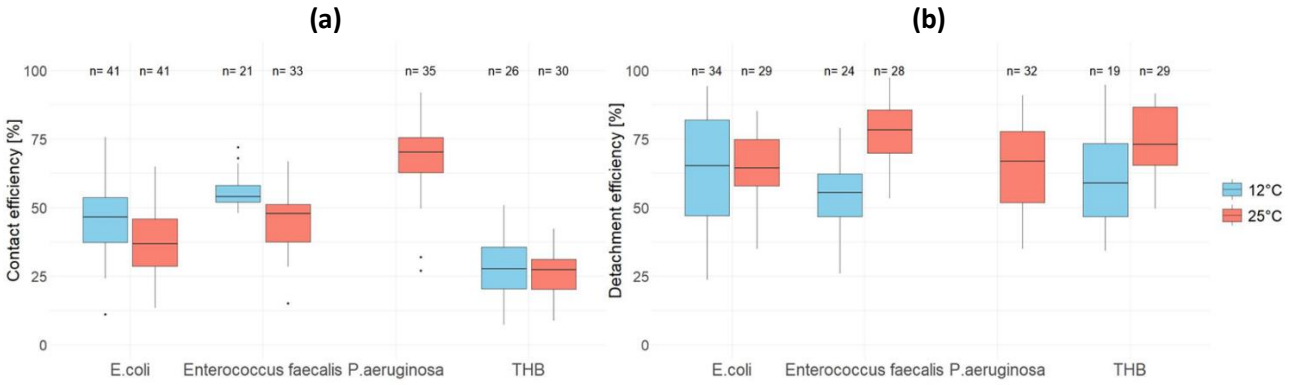

Figure 7. *Results of batch experiments with different target bacteria and water temperatures: (a) contact efficiency; (b) detachment efficiency.*

Regarding CNSs specifically developed for this application, vitality tests on *P. aeruginosa* were performed to verify they do not display any bactericidal effect. Six different CNSs were tested. *P. aeruginosa* was incubated with 50 mg of each CNS for 30 minutes at room temperature in 10 mL of PBS. SC0_C refers to TOCNF, representing the blank control of CNSs. pSC1 is the powdered form of SC1. Additionally, a flask not containing any CNS was also prepared as blank control. As shown in Figure 8, after a contact of 30 minutes the concentration of *P. aeruginosa* decreased to zero when in contact with SC0_C and SC1; CNS2 does not seem to relevantly affect negatively the vitality of *P. aeruginosa* and for this reason it was selected for further experiments. To have a more complete overview of the action displayed by CNS on *P. aeruginosa*, flow cytometry was also performed and results are reported in section 5.1.2.

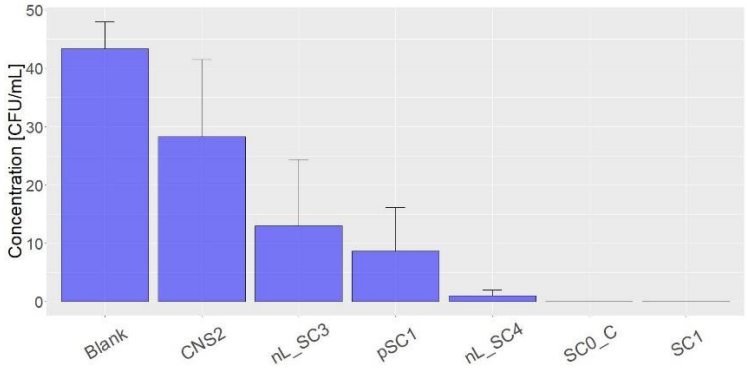

Figure 8. *Concentration of P. aeruginosa [CFU/mL] after 30 min of contact with various types of CNSs (n=3).*

The results obtained by DVGW-TUHH on *E. coli* in experiments performed at 25°C water temperature are reported in Figure 9. PAN-EDA membrane, compared to cellulose gauze, displayed a worse performance in terms of both contact and detachment efficiency.

These results, together with the ones obtained using CNS, prove that the characteristics of the material are fundamental for the successful exploitation of passive sampling. Actually, additional experiments will be performed to have more data to be used for publishing.

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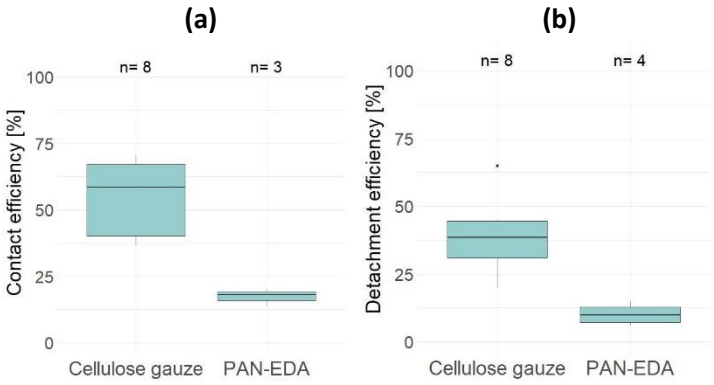

Figure 9. *Batch-scale experimental results using different passive sampler prototypes on E. coli at 25°C: (a) contact efficiency; (b) detachment efficiency.*

5.1.2 Flow cytometry

Based on results described in section 5.1.1, the effect of different CNS on cell vitality was tested by flow cytometry, using the cellulose gauze as a control for bacteriostatic effect. Results are shown in Figure 10: confirming that most of the CNS negatively influences cell vitality compared to the cellular gauze. The TCC values in the eight tested conditions were on the same level, ranging between 1.42E+05 cells/mL and 3.58E+05 cells/mL. However, as supported to the plate count methods, the nanosponge CNS2 had the smallest effect on cell vitality compared to the other 5 CNSs.

On the other side, the unexpected results about the bacteriostatic effect of CNSs on bacteria is interesting to be further investigated and exploit. In fact, a bacteriostatic material could be useful to support and integrate disinfection strategies, especially at the point of use.

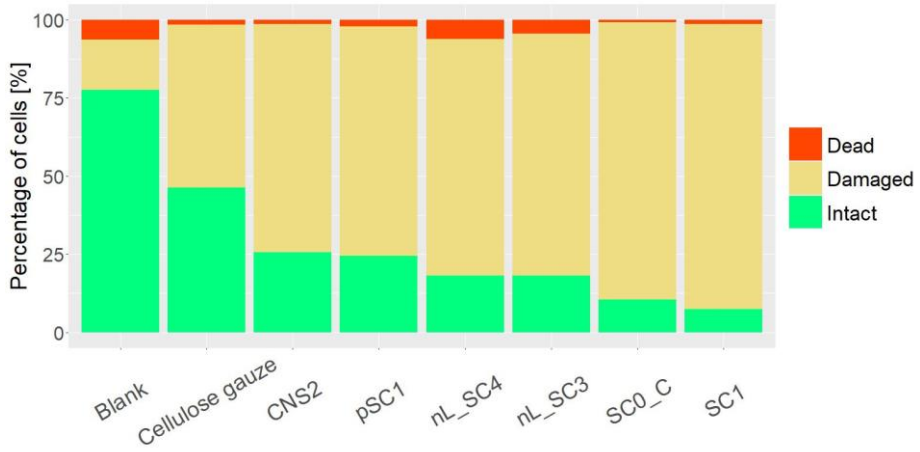

Figure 10. *Comparison of six CNSs and cellulose gauze in terms of intact, damaged and dead cells.*

5.1.3 qPCR

In addition to plate counting for bacterial quantification, qPCR was employed to provide a comparative assessment of bacterial concentration. Both methods were tested on the PAN-EDA membrane and cellulose gauze (Figure 11). The numbers are not directly comparable as *E. coli* possesses multiple copies of the 16S rRNA gene. The qPCR results were very close to the detection limit, i.e. partly outside

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the range of the standard curve. For this batch test, cultivation seems to be the more sensitive approach, but qPCR can be of advantage in real application to detect uncultivable microorganisms.

The results with qPCR agree to the trend of plate counting that the efficiency of PAN-EDA was lower than of cellulose gauze. Less bacteria attach to the PAN-EDA membrane and thus less are detached, compared to the cellulose gauze. The latter seems to be the more promising material for this purpose.

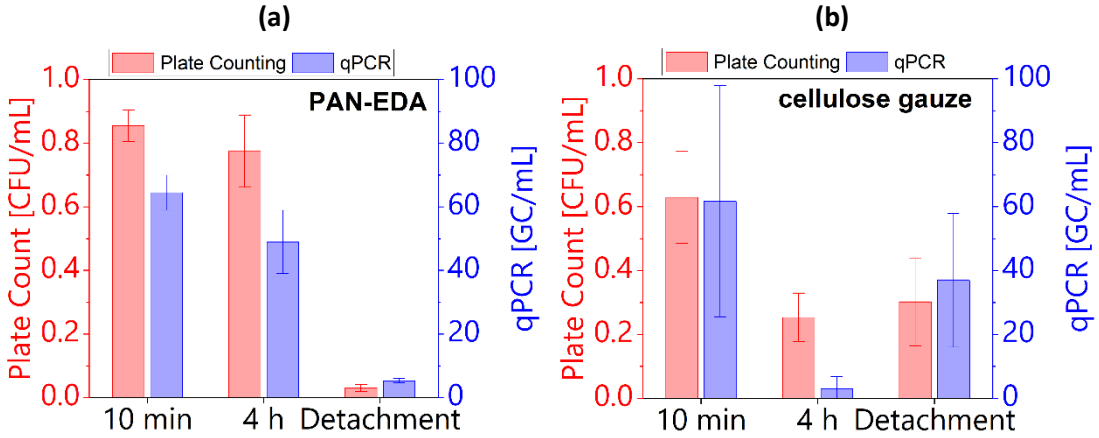

Figure 11. *Bacterial quantification using Plate Count (red) and qPCR (blue) for the attachment and detachment of E. coli on PAN-EDA (a) and cellulose gauze (b). Initial bacterial concentration measured after 10 minutes of contact time, followed by the final concentration after 4 hours of attachment, and after 2 minutes of sonication during the detachment step (n=3).*

5.2 In-flow experiment

In this section the results derived from the application of the in-flow experimental protocol are described, grouped by the enumeration method adopted.

5.2.1 Plate count

The comparison between bacterial concentrations in grab samples and in passive samplers in terms of THB and *P. aeruginosa* are shown in Figure 12.

The results of the three biological indicators were consistent with each other. The concentration of target bacteria was quite stable in the grab samples, while it increased with the contact time in passive sampler prototypes. It should be noted that *P. aeruginosa* was not detected in the grab sample collected on the 7th day, differently in the passive sampler prototype it was detected at high concentration. This confirms the results obtained in batch experiments about the good performance of the passive sampler, providing a further proof-of-concept of the potential advantages of passive sampling.

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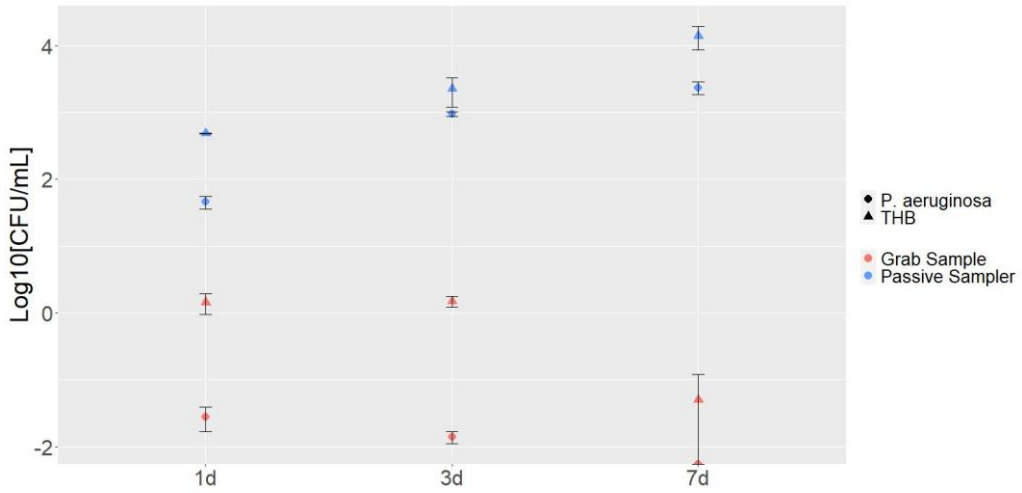

Figure 12. *Concentration (expressed as Log10[CFU/mL]) of THB and P. aeruginosa in grab samples (red) and passive samplers (blue) as a function of the contact time in the in-flow cell (enumeration by plate count).*

5.2.2 Flow cytometry

Results from the flow cytometry analysis is shown in Figure 13. The number of total cells is in the same order of magnitude in grab samples and in samples analyzed by Bactosense (~10^4 microbial/ml). Interestingly, the number of total cells detached from the sampler prototypes is one order of magnitude higher than the one in grab samples (~10^5 microbial/ml) and increases during the contact time, coherently with what observed using plate counts (see Figure 12).

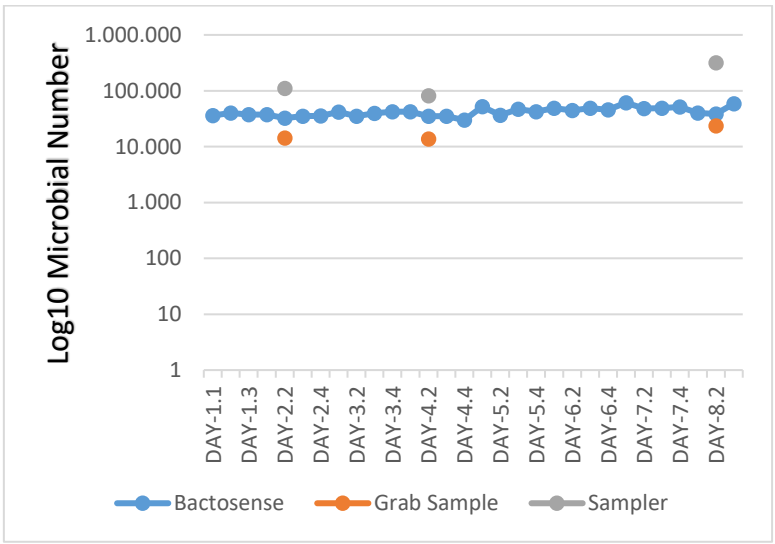

Figure 13. *Concentration (expressed as Log10[cells/mL]) of total cells in grab samples and passive samples, analyzed through flow cytometry, and samples analyzed by Bactosense.*

6. Conclusions

In this work, a proof-of concept of passive sampling for microbiological water quality monitoring was developed. In detail, two experimental protocols were defined, to evaluate the suitability of different materials to act as passive sampler for bacteria: (i) batch experimental protocol for the screening of

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the materials to be used; (ii) in-flow experimental protocol for the validation of the suitability of the selected material.

Several experiments were carried out investigating different materials (cellulose gauze, CNS and PAN-EDA) and targeting various microorganisms (*E. coli*, *P. aeruginosa*, *Enterococcus faecalis* and THB) through diversified enumeration methods (plate count, flow cytometry and qPCR). Cellulose gauze displayed the better ability to capture microorganisms, but also to permit their detachment. Water temperature affects the performance of the passive sampler, but the extent is microorganism-specific. The new materials developed within the project (PAN-EDA membrane and CNS) displayed worse performances, also showing a bactericidal effect, which might be attributed to the surface charge of these materials. In-flow experiments confirmed the possibility to use passive sampling to support an early warning system, since bacterial concentration associated to the passive samplers were higher than the ones in grab samples. In addition, passive samplers detected the presence of *P. aeruginosa* even when in grab samples it was not present.

The work performed within task 1.5 led to a couple of relevant achievements. Firstly, experimental protocols were developed to test materials to be used as passive sampler for bacteria. Then, having proved the effectiveness of this sampling methodology, it could be exploited by water utilities to support the implementation of an early warning system for detecting sporadic contaminations that are not easily captured from grab samples.

At the beginning of the project, most of the passive sampling studies on microorganisms (mainly virus) were focused on wastewater, where high levels of contamination are present. This work provided instead a proof-of-concept of the passive sampling methodology in DWDNs. The reached TRL is 3 (experimental proof-of-concept).

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