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PROOF-OF-CONCEPT EXPERIMENT REPORT

DETECTION OF BACTERIA IN AERATION SYSTEMS: FABRICATION AND SAMPLING TEST OF MICROPATTERNED SUBSTRATES OF AGAR GEL

Author: Simone Dal Zilio

Martina Conti

Istituto Officina dei Materiali - CNR-IOM

Q2 Building - Area Science Park Strada Statale 14 km 163,5 - 34149 Basovizza - Trieste

ISTITUTO OFFICINA DEI MATERIALI

Proof-of-Concept: introduction and general description

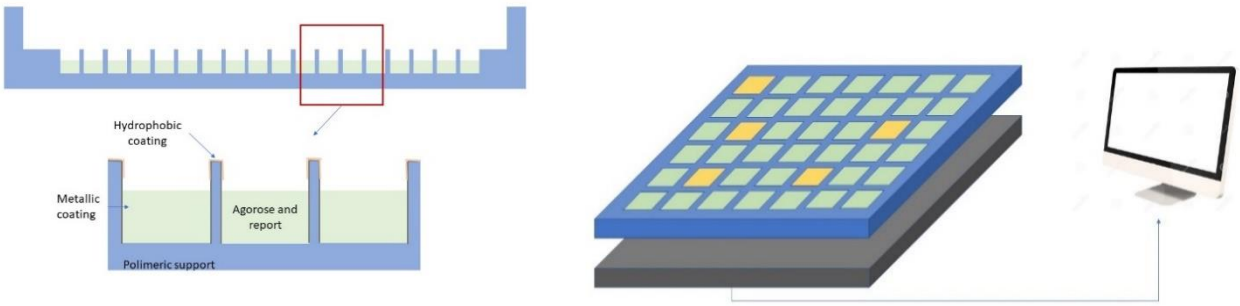
Main aim of the proposal:

A new concept of device for the research of microorganisms (bacteria), which is based on direct air sampling, with the use of a SAS (Surface Air System) sampler, will be the result of investigation. From a previous study and analysis, it has been verified that the "capture" of bacteria on the surfaces is strongly conditioned by the presence of water, also included in suitable gels. The substrates currently used for this purpose are in fact polystyrene petri dishes in which a certain quality of agarose-based gel is deposited.

The final purpose of the project initiated by the company is to have an optical detection system for the bacteria present on the device. To do this, it was hypothesized to create a “pixel” structure of the agarose substrates: these structures will then be used for the rapid count of bacteria captured during the SAS acquisition process. The pixeling of the agarose substrates is achieved through the micro-fabrication of micro-well arrays which contain an agarose gel solution; in this way they can be "coupled" to an optical detection system based on a simple CCD (Charge-Coupled device).

The gel used for the preliminary tests is LB-Agar (Luria-Bertani broth - Agar), an Agar-based gel enriched with a medium containing various nutrients, useful for promoting the growth of bacteria, and commonly used as a substrate for the bacterial counts on Petri dishes. LB-agar was chosen in such a way that the sampled bacteria, as well as adhere, can grow and form micro-colonies that can be identified within hours of sampling. For the detection of colonies, tracers or pH indicators must be add to the LB-agar, to promote a color change as a result of the growth of microorganisms. Following sampling, the micro-wells, filled with LB-agar, are placed in an incubator at 37 ° C to favor the rapid growth of any sampled microorganisms and their following identification.

In the following image, a diagram of the device and the entire system coupled to the CCD.



Work plan:

1st step: fabrication of the microstructured agar gel substrates

2nd step: Preparation of microwells for the bacteria sampling

3rd step: Sampling and bacterial detection from air samples

Measurement author and place: The fabrication process and the characterisation of each treatment has been performed by dr. Simone dal Zilio and Martina Conti at Istituto Officina dei Materiali- CNR.

REALIZATION OF PETRI CONTAINING MICRO-STRUCTURED AGAROSE

The realization of the petri dishes containing the agarose microstructures took place according to the following manufacturing process:

1. *Mould fabrication*

Starting from a silicon substrate (wafer), we defined a suitable pattern by reproducing the structures present on an optical mask on a negative resist film (SU-8 3025). The thickness of the resist is defined starting from the type of structures present on the mask: for larger structures a film with a thickness of 80 um was used, for those with higher resolution, the thickness of the resist was about 20-30 um. After exposure and development of the resist (details of the lithographic process have been omitted because they are not important for the project), the resist was thermally processed in order to harden it for subsequent replication in PDMS (SYLGARD ™ 184 Silicone Elastomer) by means of casting.

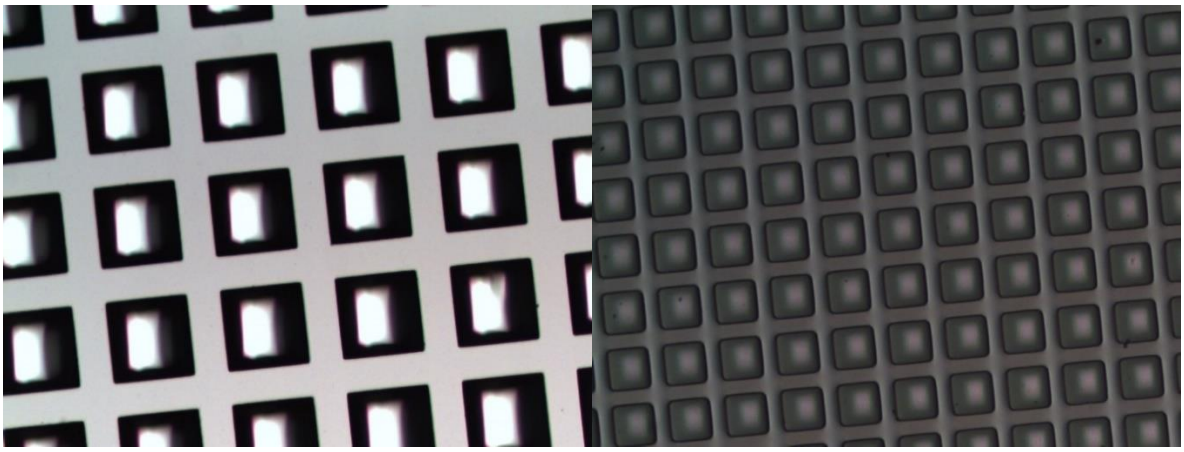
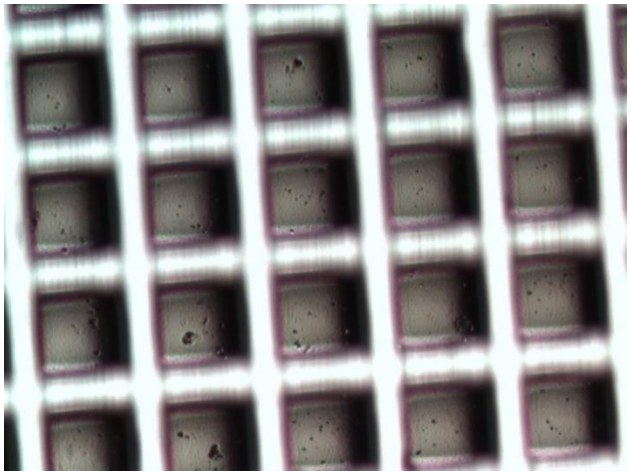
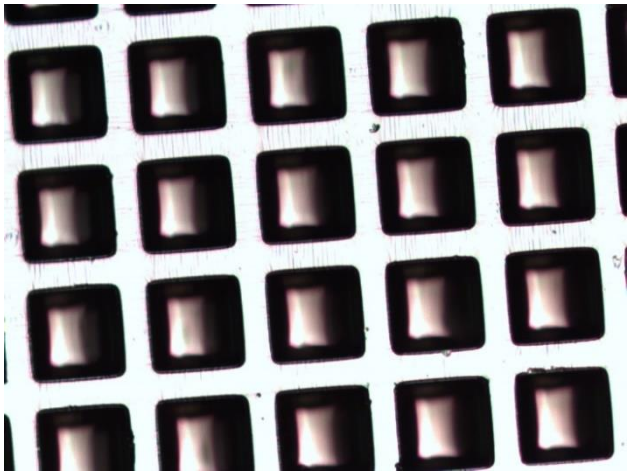


Figure 1: SU-8 mold on silicon (left) and PDMS replica (right)

2. *Polymer/Resin microstructured substrates*

The hardened silicone is used for the realization of the microstructures by means of UV-replica molding of a suitable optical resin (NOA 63 from Norland) deposited on a substrate of Polycarbonate or Polystyrene. From 1 mm thick sheets of polymer, circles with a diameter slightly smaller than that of standard petri dishes were obtained, in order to be then inserted into these. The structures obtained after exposure and subsequent removal of the PDMS mold were heat treated to obtain the final cross linking of the resin, or to make it sufficiently rigid for the subsequent filling with agar gel.

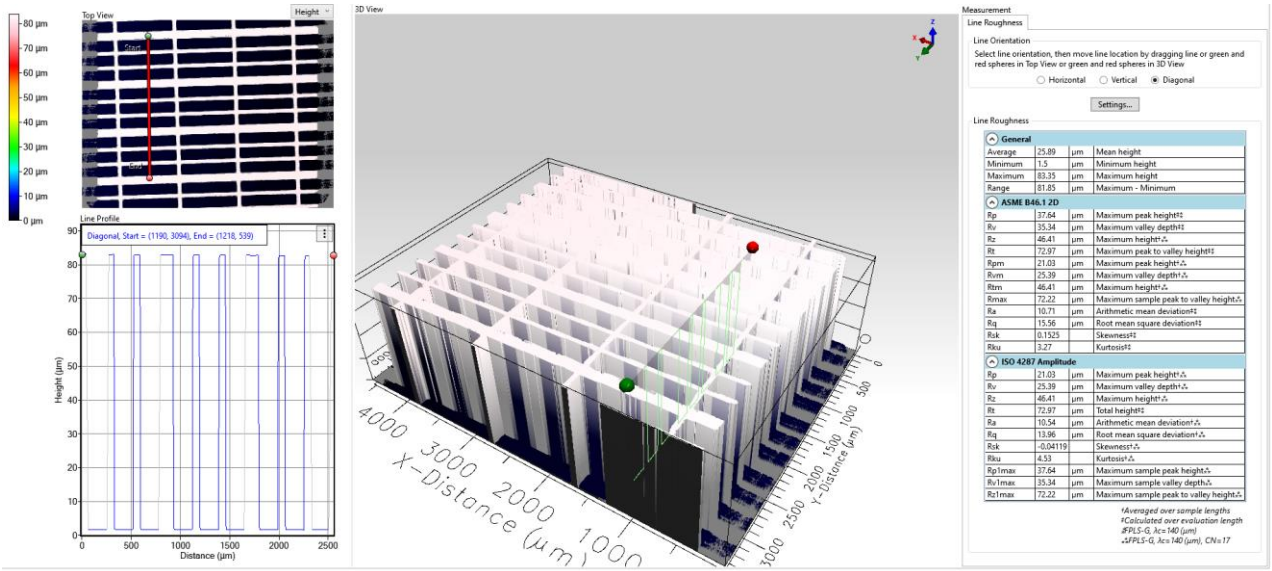




The structures created have variable dimensions (µm) as shown by the table below:

code	lateral size	periodicity	depth
25-50	25	50	60
50-100	50	100	60
75-150	75	150	60
100-200	100	200	60
500um rect	200-400	300-500	120

In the following image an example of mapping through optical profilometry of the samples:



3. Filling by agar gel

a) Filling of the wells with LB-agar gel at room T

To fill the wells with LB agar, the LB-agar solution was prepared with 1.5% agar and 2.5% "LB, Miller" in milli-Q water. Once ready, the solution was subjected to autoclaving (120 ° C for 20 min). At room temperature, the solution changes from a liquid state to a gel state. To fill the wells, the ambient T gel was pressed onto the surface of the wells.
Given the inefficient filling, the gel was prepared by decreasing the agarose concentrations from 1 to 0.1%. Although the filling was improved, it was still not sufficient for the complete filling of the wells, possibly due to a "swelling" effect of the gel on the surface.

b) Filling of the wells with liquid solution of LB-agar at T=60 ° C

We decided to fill the wells with the liquid solution of LB-agar at 60 ° C, by placing the micro-wells directly on a heated plate during the filling process. This procedure improved the filling of the wells, although the process was not yet optimized.

c) Treatment of the surface of the wells with Plasma oxygen

To improve the filling, the surface of the wells was subjected to a 20W plasma oxygen process for 2 minutes, in order to increase their wettability. Thanks to the treatment of plasma oxygen in combination with the use of the liquid solution of 0.1% agar, a further improvement in the filling of the wells was observed.

d) Filling of the wells with liquid solution of LB-agar (0.1% agar and 2.5% LB) at room T on microwells treated with O2 plasma

The optimization of the filling procedure has been reached first by treating the microwells with plasma oxygen, as described before, then filling them with the LB-agar solution at T>70°C (after autoclaving procedure) at room temperature.

In the following image, an example of the ultimate microwells after filling procedure:

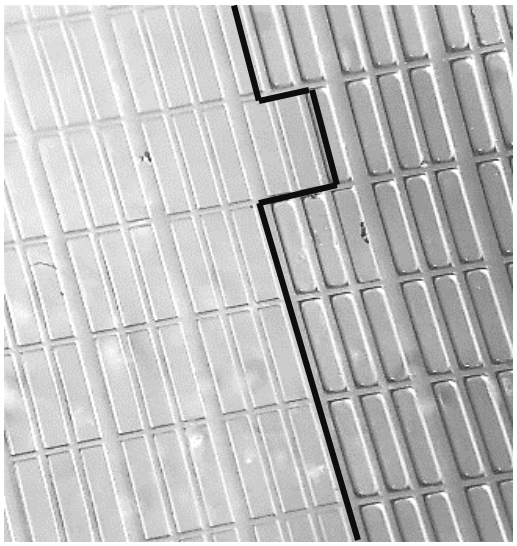


Figure 2: Image of microwells after filling with LB-agar. The black lines shows the interface between the unfilled (on left) and the filled zone (on right)

PREPARATION OF MICROWELLS FOR THE BACTERIA SAMPLING

As the device must be made sterile to avoid any contamination from the sample, the final substrates after plasma O2 were soaked in 70% EtOH / Water-milliQ solution for 20 min then rinsed in milliQ water and allowed to dry under a laminar flow hood. Once dry, the micro-wells were filled at room temperature under hood with a solution of LB (2.5% w / v) - Agar (0.1% w / v), previously autoclaved at 120 ° C for 20 min. After filling, the substrates were adhered to the bottom of a 90mm Petri dish via an adhesive layer of PDMS.

In the following, an image of microwells filled with LB-agar gel and placed on the bottom of a 90 mm Petri dish:

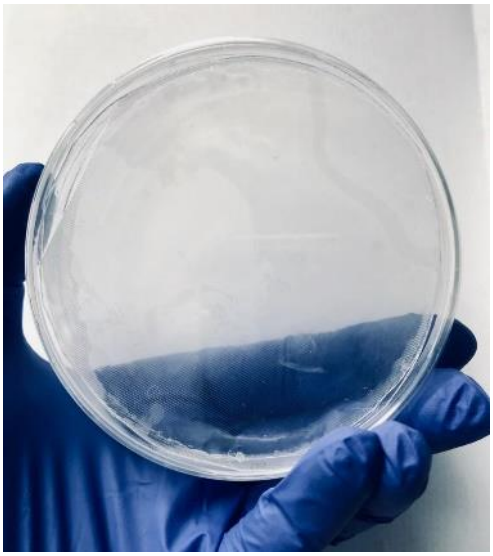


Figure 3: Microwells filled with LB-agar gel and placed on the bottom of a 90 mm Petri dish

As sealed, the samples at 5-8° C last up to 2 weeks from the preparation. In the incubator, at 37°C and humidified environment, we observed a drying time of 2 weeks.

SAMPLING AND BACTERIAL DETECTION FROM AIR SAMPLES

The microwell substrates placed on a 90 mm petri dished were sealed with parafilm and placed at 5-8°C until use. Once the effective adhesion has been ascertained, we performed the sampling test on microstructured substrates filled with agarose gel. In order to test our samples, two different microwells placed into Petri dish have been sent to “Work in Progess Bio medical” where air sampling with SAS has been performed before and after peroxide treatment. After 24h from sampling, we characterized the samples by optical microscopy and the results showed the effectiveness in the bacteria adhesion on our substrates.

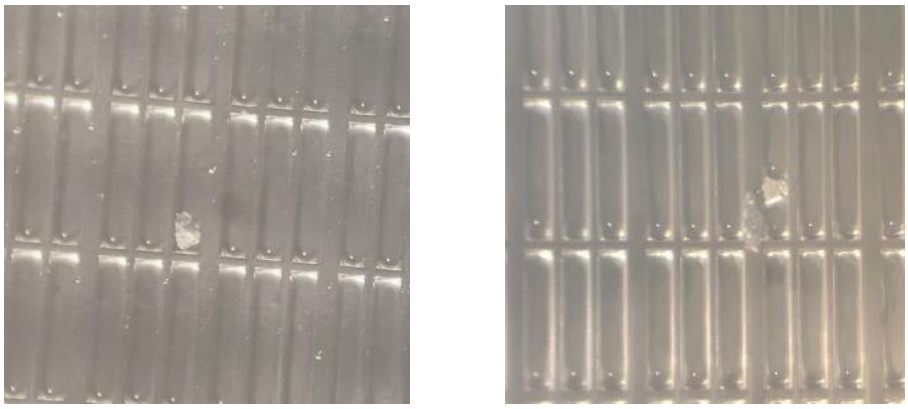


Figure 4: Microwells filled with LB-agar gel after 24h from air sampling with SAS

NucBlue staining on the samples and following fluorescence images confirmed the adhesion of living bacteria.

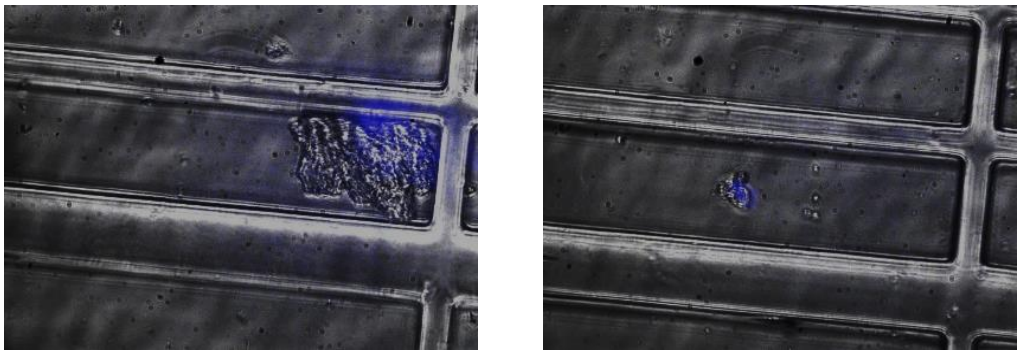
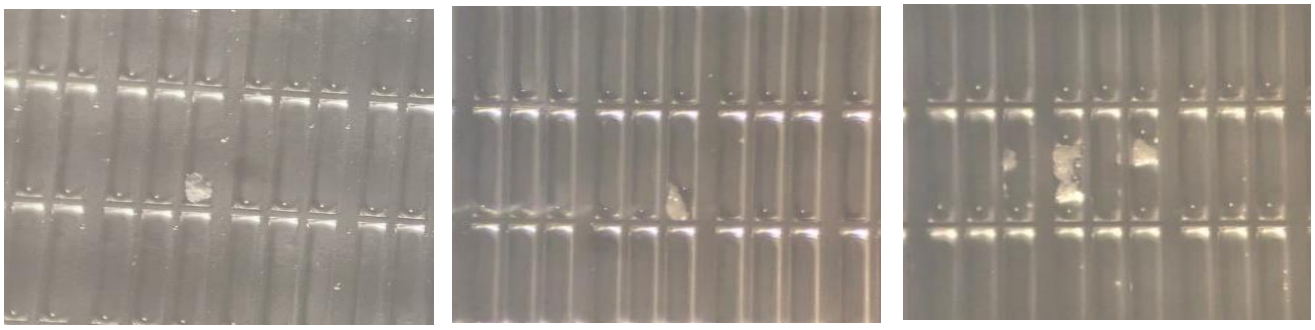


Figure 5: Fluorescence images of bacteria colonies adhered on microwells. Living bacteria were identify by NucBlue staining.

After sampling, the substrates were incubated at 37°C and the bacteria growth has been followed over time:



After 24h After 48h After 72h

Figure 6: the image shows the bacterial growth over time: after 24h, 48h and 72h.

The analysis of bacteria colony counting on micropatterned substrates performed by optical miscopy showed:
- after 24h by sampling, a number of 8 CFU and 3 CFU for substrates before and after peroxide treatment, respectively.
- after 72h by sampling, a number of 28 CFU and 4 CFU for substrates before and after peroxide treatment, respectively.

The results demonstrated that our substrates allow the adhesion and growth of bacteria. Moreover, these values are in agreement with the bacteria counting results performed on Petri dish before and after peroxide treatment by the microbiology laboratory (Testing Point 3 s.r.l.), as showed in the Fig.8

Descrizione del campione: 1 Magazzino-Prima #
Temperatura di arrivo (°C): 4,0
#: Informazione fornita dal committente
Data Accettazione: 04/02/2022 12.48 Data inizio prove: 04/02/2022 Data fine prove: 07/02/2022

DESCRIZIONE ANALISI	U.M.	RISULTATO	NOTE
Conta di Microrganismi vitali a 37°C Rapporti ISTISAN 2013/37 Pag 49	ufc/piastra	22	

Descrizione del campione: 2 Magazzino-Dopo #
Temperatura di arrivo (°C): 4,0
#: Informazione fornita dal committente
Data Accettazione: 04/02/2022 12.48 Data inizio prove: 04/02/2022 Data fine prove: 07/02/2022

DESCRIZIONE ANALISI	U.M.	RISULTATO	NOTE
Conta di Microrganismi vitali a 37°C Rapporti ISTISAN 2013/37 Pag 49	ufc/piastra	5	

Figure 7

Conclusions and final remarks

The purpose of the PoC presented was to highlight the possibility of creating microstructured systems useful for the capture of sampling bacteria from the air using the SAS technique. The proposed solution, consisting of micro-well arrays filled with agarose, was developed and samples with different geometries were made and characterized by optical microscopy and SEM.

The validation of the devices from the point of view of bacterial affinity was carried out and validated through fluorescence microscopy techniques.

The results obtained show that the proposed technology is effective for the company's application, opening up the possibility of subsequent implementations of a similar bacterial detection technology.

This report has been written on 24th June 2022