



*Research article*

## **Suitability of Rose Bengal sodium salt staining for visualisation of face mask contamination by living organisms**

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**Abstract:** Unworn masks and masks provided to us after having been worn conformable to law (mandatory wearing of masks) served as test objects. In order to identify the distribution of living microorganisms on the surface of a mask dependent on exposure time and distance from the human face we conducted a staining study using the bengal rose method. The regular deposition of living microorganisms on artificial mask surfaces was more intense in the areas close to the mouth and nose. A time dependent accumulation was larger on the inside in comparison to the outside of the mask, even if the mask was not worn but only left in the room. The most interesting finding was the ability of microorganisms to penetrate all layers of the mask. We therefore conclude that masks are a suitable substrate for the cultivation of germs, even when not worn. Colonisation increases with human use and with time.

**Keywords:** bioadhesion; Fluorescent staining; personal protective equipment; masks; surgical mask; microbial contamination.

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### **1. Introduction**

Since the SARS-CoV2 outbreak in 2019, masks have become mandatory in most countries around the world for the general population. They have become the ubiquitous piece of fabric worn in the face.

Contamination of masks with bacteria and fungi is well known fact [1,2]. Several microbiological methods exist to track, quantify and estimate microbial contamination of face masks, which are of a

porous structure. To our knowledge so far there has not been a validated approach to visualise the real macro and microscopic mask contamination effectively.

Our idea was to test the suitability of the rose bengal staining method for visualising the colonisation in masks. Rose bengal sodium salt, a chemical compound (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) with the sum chemical formula  $C_{20}H_4Cl_4I_4O_5$ , is applied to assess and visualise the extent of vital microbial presence in porous structures. This dye is used in biology as well as medicine for visualizing living bacteria, fungi and human cells. To date, there have been no studies on the use of Rose Bengal for staining viruses to our knowledge. During the 1950's rose bengal was used in the staining procedure of foraminifera. The advantage of such staining samples is that living organisms at the time of sampling can be distinguished from dead organisms that do not stain [3]. The use of bengal rose to stain samples of porous structures allows one to determine the extent of penetration of organisms into the structure, the area where they accumulate as well as the easier identification of small organisms (bacterial colonies, fungi, lichens) and invertebrates [4]. Rose Bengal is generally used to detect organisms that are alive at the time of sampling. From many years of experience in staining biological samples containing live and dead adult aquatic invertebrates (meiobenthos) and detritus, it is clear that organisms that were dead at the time of sample collection do not stain at all. Detritus is also not stained, but if it is colonized by microorganisms (e.g. bacteria) total areas of it will stain. But in this case we are again dealing with the staining of living organisms that have colonized the dead organic remains. The presented staining method has already been applied to various porous structures including hydrotechnical concrete [5,6].

But, to our knowledge, the presented methodological staining study is the first to test the suitability of bengal rose for visualising mask colonisation.

The aim of the study was to identify the distribution of microorganisms on the mask surface as a function of exposure time to environment and distance from the human face.

## 2. Materials and methods

Common surgical face masks (as required by law to contribute to the containment of SARS-CoV2) were used for the staining study. The disposable medical face masks measured 175 mm x 95 mm, consisted of three layers and were of the type IIR. The staining study included the examination of a mask after having been worn conformable to law (mask obligation). The mask was provided to us after having been worn for 1, 2, and 5 hours, respectively (Setting 1). Unworn masks served as a control.

In another environmental setting, masks were exposed in a room of 25 m<sup>2</sup> with a ceiling height of 2.5 m filled with 10 people (Setting 2). The masks hung at a height of 2 m above the floor. This second experiment on masks lasted 1, 2 and 10 hours whereby the people left the room after 5 hours. The room was neither air-conditioned nor intensely ventilated.

In a third setting the inner side of the mask was placed 1 m away in the direction of the speaking person (reading a book) for 1, 2 and 5 hours (Setting 3). The human subject was healthy (no coughing or sneezing) and was advised not to scream or laugh violently. The control mask was placed 5 m away from the face. The large experimental room (40 m<sup>2</sup>) had been aired every hour for 5 minutes.

In the fourth setting, a mask was worn in compliance with the law (mask obligation), but was reused several times before it was handed over to us for further examination. After each use for about 3 hours per day for 2 weeks, it was kept in a bag by the wearer (Setting 4).

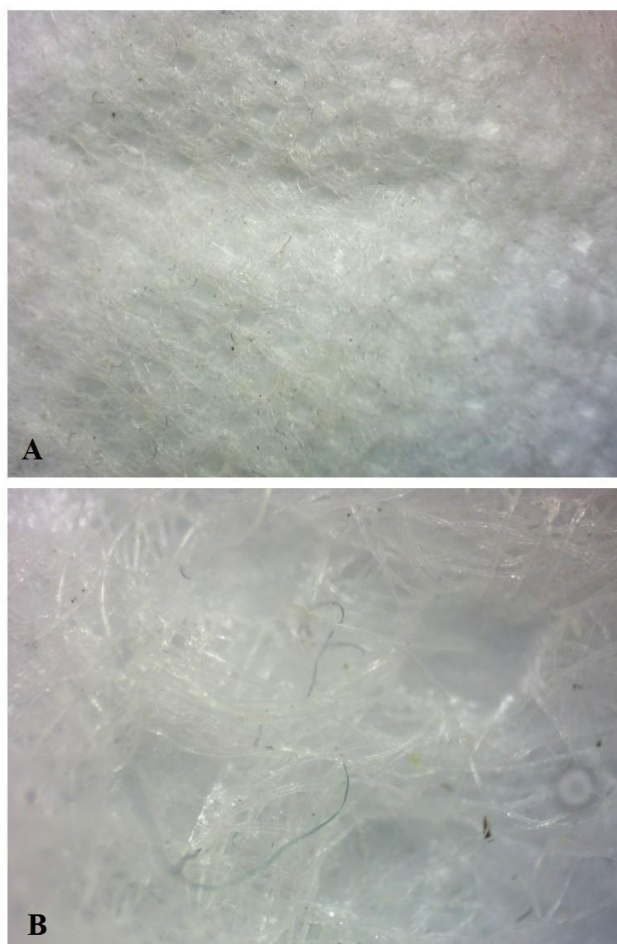
After each completed treatment of the masks (Settings 1-4), the masks were immediately preserved in 70% ethanol. Thereafter they were stained for qualitative and partly quantitative analyses (staining intensity). A solution of 0.1 g bengal rose sodium salt (SIGMA No. R3877-5G) / 0.5 l distilled H<sub>2</sub>O was used to stain the masks. The masks were stained for 10 hours in bengal rose solution. Excess dye was gently rinsed off.

Macroscopic photographs were taken of both surfaces of the mask (externally and internally).

Microscopic images were made of selected areas when masks had been worn: the area around the mouth and nose, the edge of the mask adjacent to the nose and the chin. The magnifications were 7x and 30x.

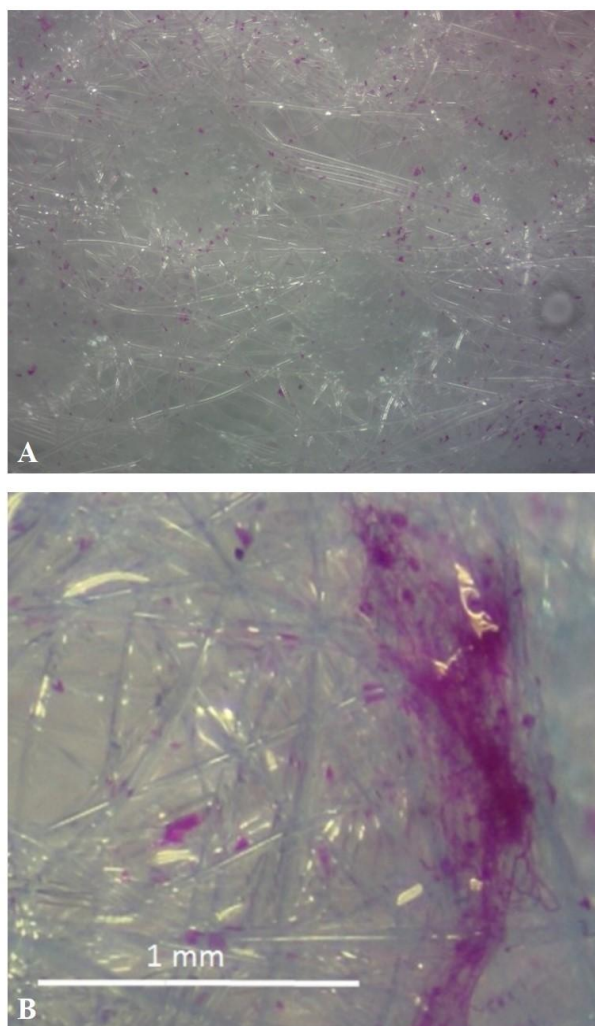
### 3. Results

The procedure used allowed us to determine the distribution and intensity of living microorganisms on the mask surfaces depending on distance and wearing period. From the images obtained it was possible to trace – in a quick and relatively simple way – the process of microbial deposition on different surfaces of disposable masks. Mask appearance as taken out of the package is illustrated in Figure 1.



**Figure 1.** A - Clean, new mask as taken out of the package: view of inner surface structure. B - higher magnification: dark, loose fibres that are not an integral part of the mask structure are visible.

Figure 2 shows how the microstructure surface of a worn mask had been contaminated with living microorganisms, which stained intensely pink (Figure 2A). Viable microorganisms were also found between the fibres of the inner area of the mask (Figure 2B).



**Figure 2.** Example image of bengal rose stained living structures (microorganisms, cells) on the surface and between the fibres of a worn face mask: A depicts fine structures scattered on the surface and between the fibres of the mask, B shows clusters of microorganisms between the scattered fine living structures.

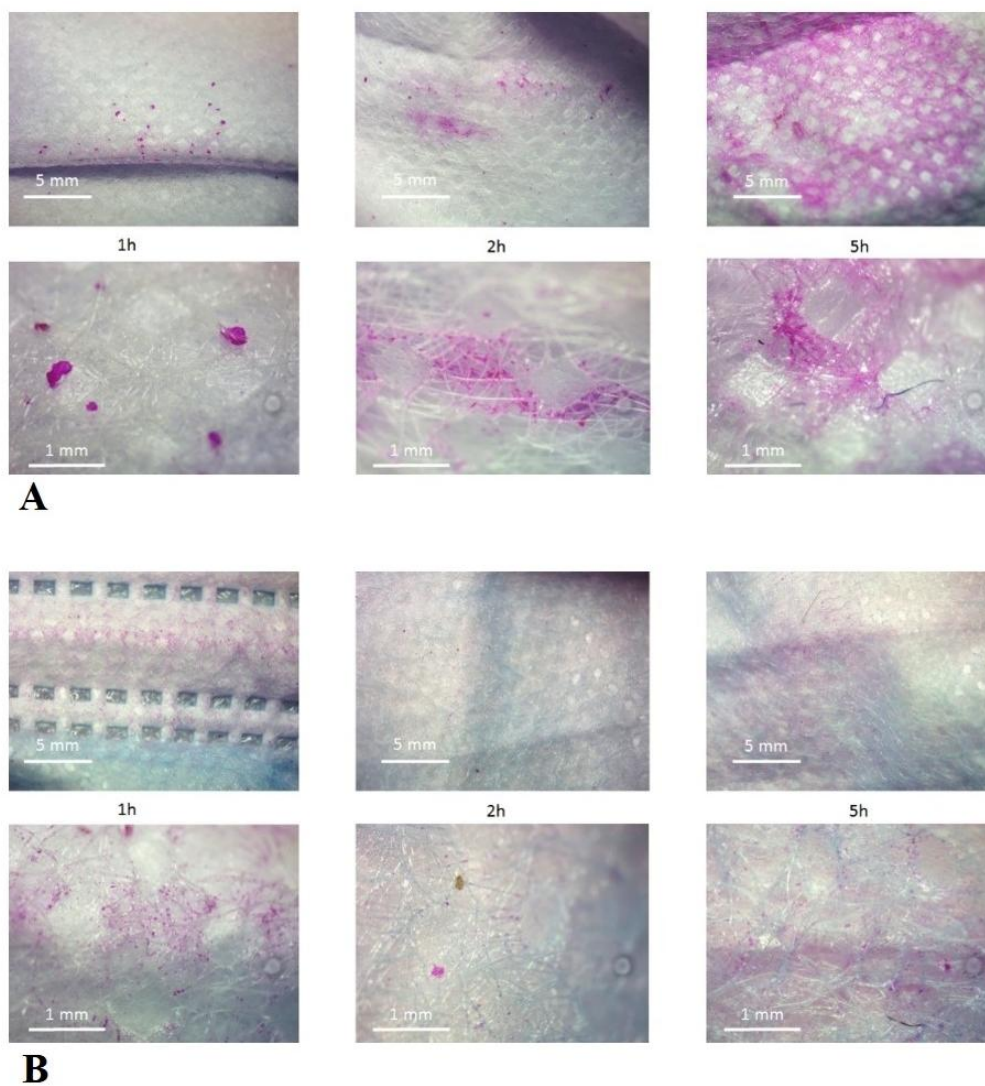
In Setting 1, a mask was evaluated that was worn for up to 5 hours. The results showed that from a longer exposure to wearing a mask (1, 2 and 5 hours) there was an increase in accumulation of living microorganisms on the inner surface of the mask indicated by a continuously stronger staining. (Figure 3). The accumulation of living microbes appear predominantly in the dominant part of the mask representing the area around the mouth and nose (Figures 3A and 4A). The possibility of microorganisms penetrating through all mask layers is shown in Figures 3B and 4B. A cluster of significantly stronger stained structures were found in the same part of the mask on the inside as well as on the outside. The concentration of microorganisms on the inner surface of the mask in Figure 3

was caused by sneezing. The pressure thereof may account for the penetration of microorganisms through the structures of the mask. However, this observation requires further detailed research.



**Figure 3.** Macroscopic images of stained masks from Setting 1 (masks worn). A=inside, B= outside view of the mask.

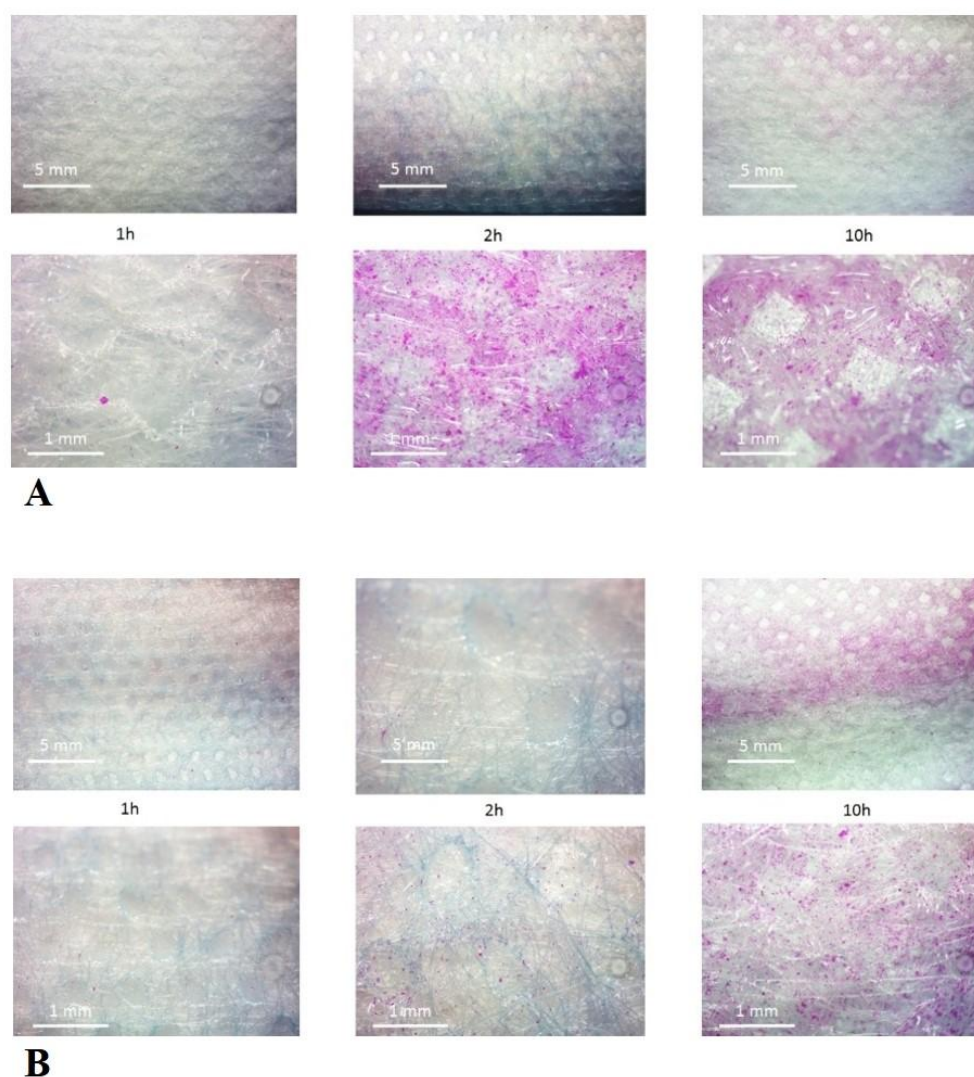
Macroscopic images of the masks from Setting 2, which hung in a crowded room at a height of 2 m helped to depict the deposition of microorganisms on the inner (Figure 5 A) and outer (Figure 5 B) surfaces of the masks. This happened without direct human interference on the mask, which was used as a culture medium. Interestingly, any number of microorganisms could be deposited on both sides of the mask because the mask was suspended at a height of 2 m and not placed on a person's face. The image obtained after a 10 h exposure indicates that an increased deposition occurred on the inner side/white side of the surgical mask (Figure 5), which is less hydrophobic than the bluish surface. Microscopic images confirm this remarkable observation (Figure 6).



**Figure 4.** Microscopic images of stained masks from Setting 1 (masks worn). A= inside, B= outside view of the mask.



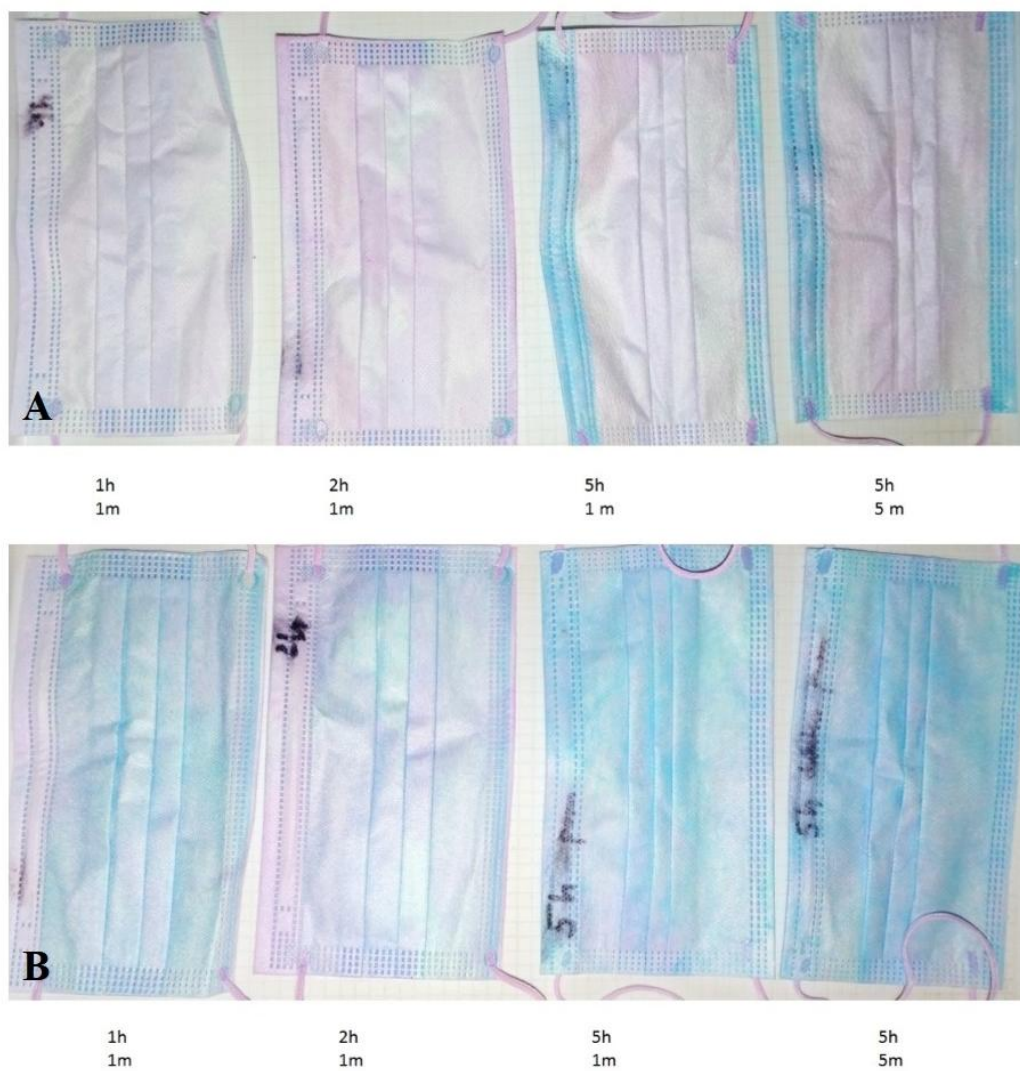
**Figure 5.** The photographs show the inner and outer surface of stained masks from Setting 2 after different exposure times: A=inner part, white, B=outer part, blue.



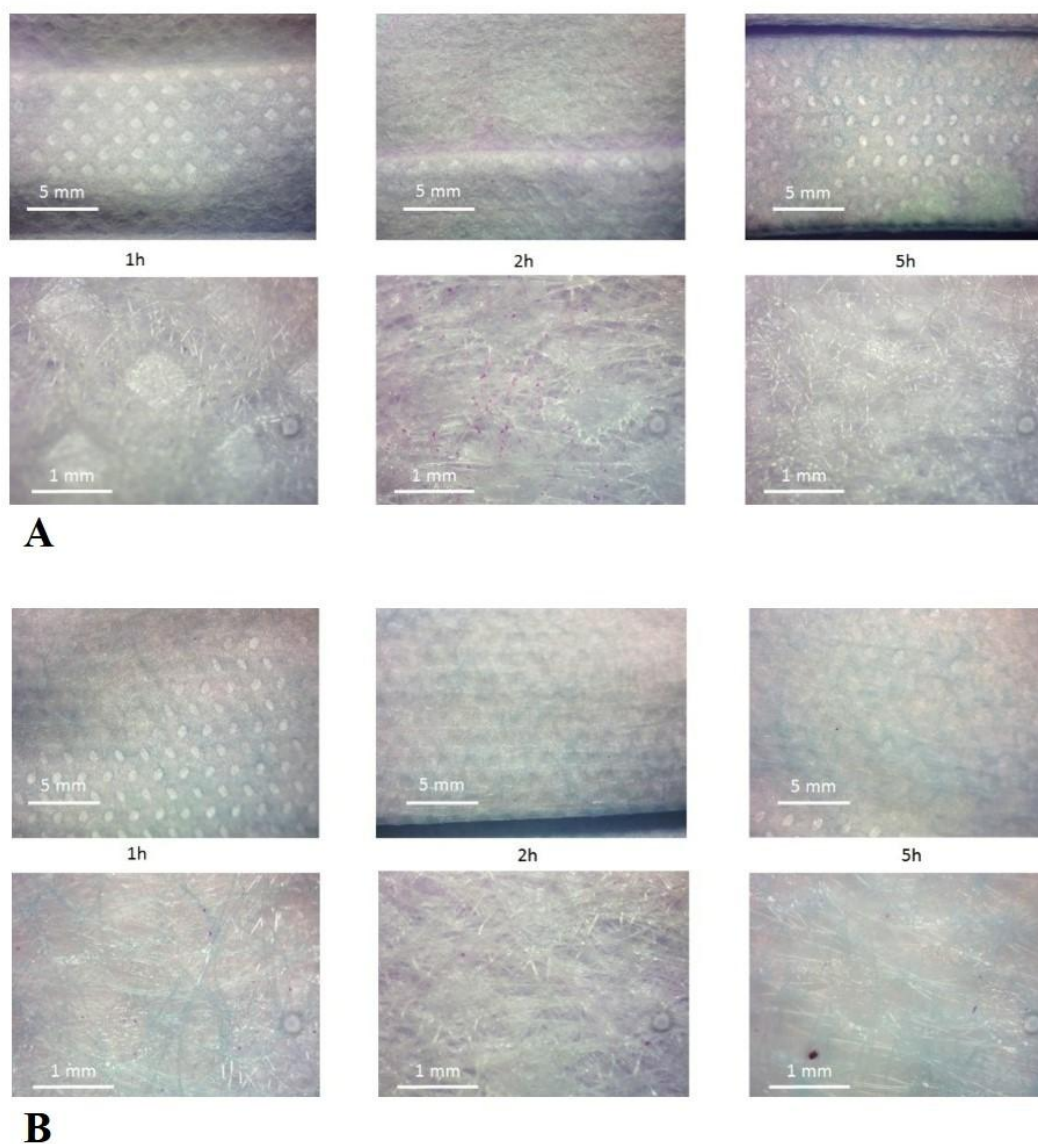
**Figure 6.** Microscopic image of the stained masks from Setting 2 that hung at a height of 2 m in an unventilated crowded room of 25 m<sup>2</sup> accommodating 10 people after exposure of 1, 2 and 10 h: A shows the inner layer of the mask, B depicts outer layer of the mask.

In Setting 3 the results obtained indicate a low density of microorganisms/living structures on the surface of the mask (Figure 7). The distance of 1 m from the face, a relatively large room and its systematic ventilation counteracted the accumulation of microorganisms on the mask (Figure 8). The most abundant accumulation of microorganisms on the mask was observed after a 2 h exposure, which might indicate a randomized event. After a 5 h exposure the number of microorganisms is comparable to the mask exposed for 1 h (Figure 8).





**Figure 7.** Macroscopic image of the stained masks after exposure of 1, 2 and 5 hours at the distance of 1 m and 5 h at the distance of 5 m from the face (a person without the mask reading a book aloud) in a room ventilated every hour ( $40 \text{ m}^2$ ): A= inner side of the masks, B= outer side of the masks.

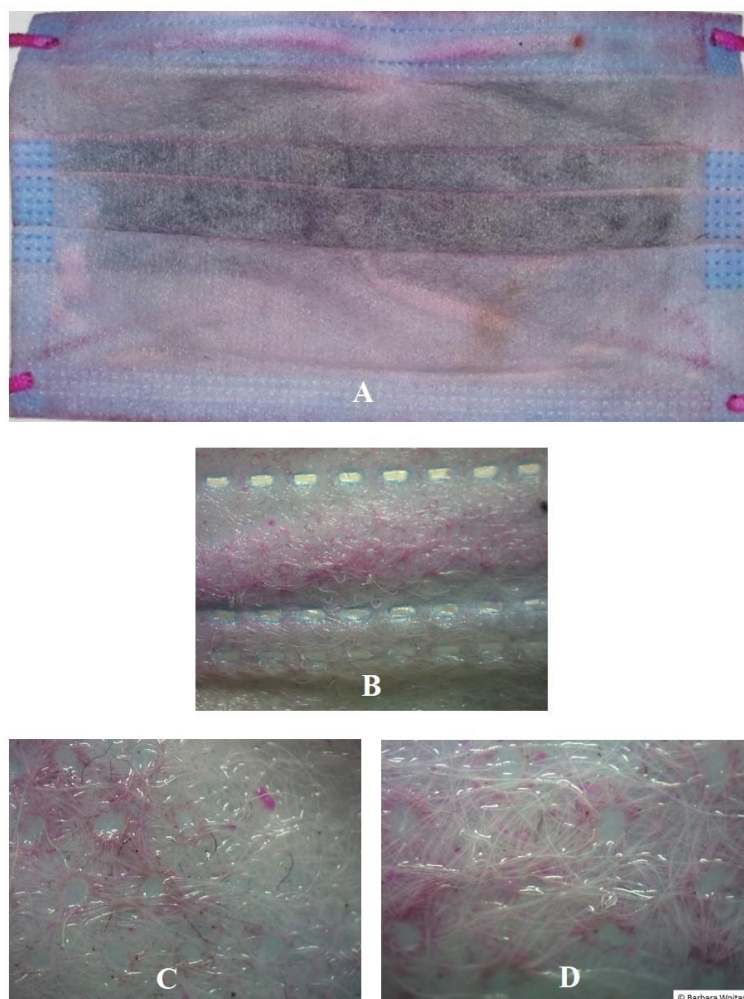


**Figure 8.** Microscopic image of the stained masks after Setting 3 with exposure of 1, 2 and 5 h at a distance of 1 m from the face (a person without a mask reading a book aloud) in a room ventilated every hour ( $40 \text{ m}^2$ ): A= inner side of the mask, B= outer side of the mask.



**Figure 9.** Setting 3: Microscopic image of the masks after exposure for 5 h at a distance of 5 m from the face (a person without a mask reading a book aloud) in a room (40 m<sup>2</sup>) ventilated every hour: Left pictures = inner side of the mask, Right pictures = outer side of the mask, single clusters of microorganisms (coloured intensely pink) are visible.

In Setting 4, a repeatedly used mask was evaluated (2 weeks, about 3 hours a day, the rest of the time it was stored in a pocket). After staining the main part of the mask was dark in colour, which occurred after the staining with rose bengal despite the mask being dirty (Figure 10 A) - it was not a brand new white. The microscopic image shows compact living structures overgrowing the structure of the mask (Figure 10B,C,D). The lighter colour of some of them however may indicate necrosis. In this case they create the foundation for new microorganisms, which results in the formation of microbial cultures in the mask.



**Figure 10.** Image of the mask after two weeks of use for about 3 h a day, for the remaining time the mask was in the pocket: A is a macroscopic image, while B, C and D are microscopic images. Shown is the region of the mask adherence to the bridge of the nose (B), the centre of the mask - mouth and nose region (C and D) - visible substrate with poorly stained structures (lighter colouring), partly dead structures where intense pink dots are visible (living microorganisms).

#### 4. Discussion

The chosen staining method is a novel approach that allows the assessment of living microorganisms in the environment. Up until now it has been used for biological scientific purposes. Bengali rose dye enables to determine the prevalence of living microorganisms on artificial surfaces [6]. Our findings are a valuable tool for the assessment of mask contamination and its use. The warm and moist environment created by worn masks paves the way for microorganisms to grow unhindered [7]. Artificial mask surfaces do not have protective mechanisms like antibodies, complement system and defence cells that are usually part of a vital human mucous membrane. The biological consequence is an ideal breeding ground for various pathogens such as bacteria and fungi [8,9]. The germ density is proportional to the time the mask is worn. Experimental studies

observed that after wearing the mask for only 2 h the pathogen density increased nearly tenfold [10]. This scientific data corresponds to our findings. Time could be a proven variable responsible for the accumulation of living organisms in our experiment as well. Moreover, the hydrophilic inner mask surfaces contribute to better humid growth conditions even if not worn in the face. The outside hydrophobic layer of a surgical mask is a nonwoven polypropylene sheet less optimal for microorganism growth. Polypropylene is a naturally hydrophobic polymer and causes water to bead-up on its surface. In contrast, the inner side is hydrophilic [11]. Such a three-layer mask partitioning with a hydrophilic inner and a hydrophobic outer layer is recommended by the WHO [12]. However, we found the hydrophilic inner layer to correlate with a higher contamination of living organisms. With the interesting images obtained from these tests important questions arise: Could microorganisms deposited on the inside of the mask re-enter the body via breathing, especially the respiratory tract? And if so, to what extent?

## 5. Conclusions

The bengali rose sodium salt staining method is a novel approach that allows the assessment of microorganisms in the environment and has been used for biological scientific purposes thus far. This staining enables to determine the prevalence of living microorganisms on artificial surfaces. According to our results it may be a valuable tool for the assessment of mask use. Our findings and the excellent visualisation indicate the suitability of this method for the experimental assessment of microbial contamination of face masks. We could confirm the regular deposition of living microorganisms occurring in the microstructures of the mask by means of the intensity depending on exposition time while wearing a mask and its contact to the breathing orifices.

A time dependent accumulation was larger on the inside than on the outside the mask, even if the mask was not worn. This was most probably facilitated by the hydrophilic properties of the inner layer of the mask in contrast to the hydrophobic outer layer. The ability of microorganisms to penetrate all layers was the most disturbing finding. The mask is capable to be a suitable substrate for the cultivation of germs even when not worn. The colonisation increases with the wearing period and with time.

## Acknowledgements

Author Contributions: Conceptualisation, B.W. and K.K.; methodology, B.W., software, B.W., K.K., formal analysis, B.K., K.K.; investigation, B.W., K.K.; writing—original draft preparation, B.W., K.K.; writing—review and editing B.W., K.K.. All authors have read and agreed to the published version of the manuscript. We thank Bonita Blankart for proofreading the manuscript. Funding: This research received no external funding.

## Conflict of Interest

The authors declare no conflict of interest.

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