D4.1.1.1, D 4.1.1.2, D 4.1.1.3

Wastewater treatments

Photodisinfection

PROJECT AdSWiM

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	Clara Comuzzi		

INTRODUCTION

As already mentioned in the previous reports, photodynamic inactivation (PDI) is receiving great attention as a promising wastewater treatment technology, owing to its environmental-friendly characteristic, low-energy consumption, and low-cost inputs. PDI is a process that uses oxygen dissolved in water as the disinfectant agent. PDI only takes place when a photosensitizer (PS), light with appropriate wavelength (coincident with one of the photosensitizer maximum absorption peaks) and molecular oxygen are present. PS is a molecule acting as an intermediary agent that can absorb the energy of the light source and then transfer, to the dissolved oxygen, either electrons (type I mechanism) or energy (type II mechanism) to generate reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide, free radicals like superoxide and hydroxyl radical. Resultantly, ROS attacks cellular DNA, RNA, proteins, and lipids, leading to the destruction of cells. Therefore, PDI is a multi-target sterilization technology making pathogens difficult to develop resistance (Bartolomeu et al., 2017; Li et al., 2021).

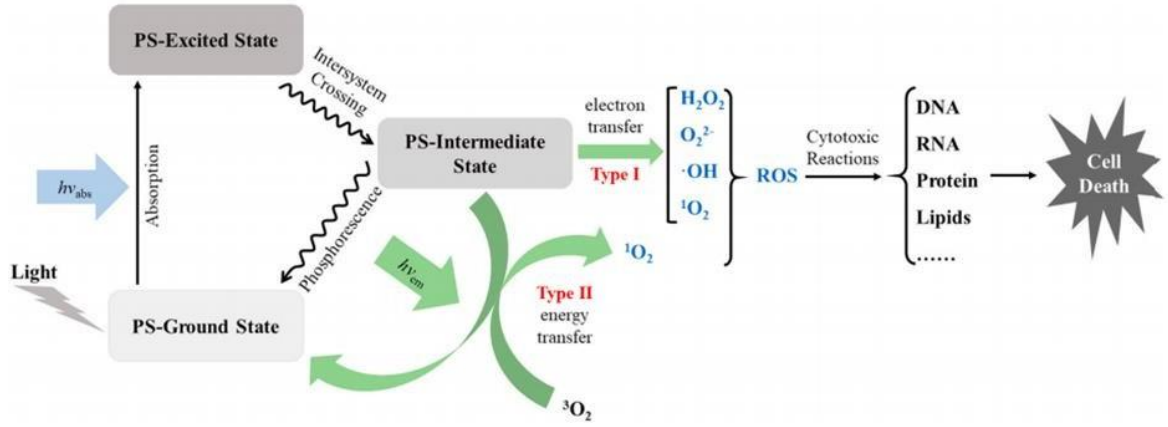


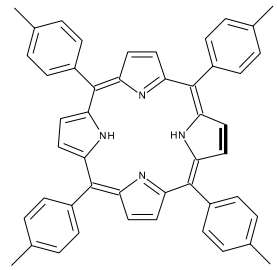
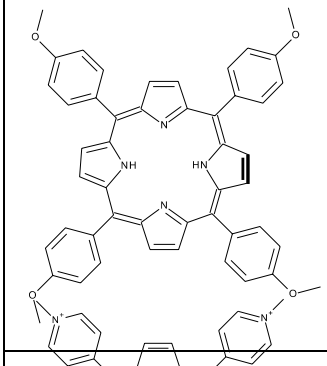
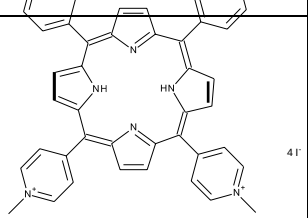
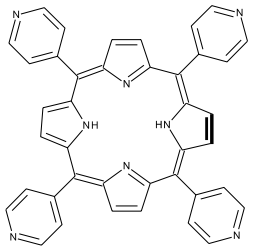
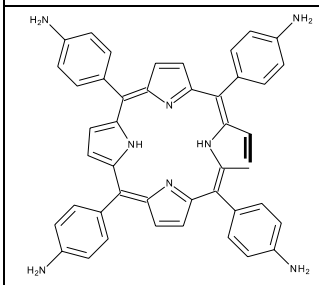
Figure 1 ROS generation by PDI via type I and type II photodynamic mechanisms (Li et al., 2021).

The antimicrobial photodynamic process shows many positive aspects including a broad spectrum of action, efficient inactivation of antibiotic-resistant strains, and low mutagenic potential (Zoltan et al., 2015).

Porphyrins are one of the most studied and described photosensitizers in the scientific literature, but, nowadays, the use of porphyrins as a photosensitizer for large-scale applications is made possible as the synthetic production processes have been significantly improved, bringing on the market a variety of porphyrins at reasonable prices. Consequently, commercial porphyrins were chosen (Table 1) in this project as photoactive components for the synthesis of photoactive materials devoted to water disinfection. The previous work, summarized in Report 2, showed that polyvinyl chloride was a suitable polymer for the synthesis of photoactive material for water treatment. The film dopped with

different porphyrins was able to generate singlet oxygen in water solution by irradiation with white light. Moreover, the film was chemically stable under working conditions, thin and flexible, and obtained by a single synthetic step.

Table 1 Commercial porphyrins used in the experiments

Molecular structure	Commercial name	Acronym	Number
	5,10,15,20-(tetra-p-tolyl)porphyrin	4MeP	**1**
	5,10,15,20-(tetra-4-methoxyphenyl)porphyrin	4OMeP	**2**
	5,10,15,20-(tetra-N-methyl-4-pyridyl)porphyrin tetraiodide	4MePyP	**3**
	5,10,15,20-(tetra-4-pyridyl)porphyrin	4PyP	**4**
	5,10,15,20-(tetra-4-aminophenyl)porphyrin	4NH_2P	**5**

Data on the photodynamic activity of porphyrins versus *S. aureus* (Report 3) highlighted that porphyrin with a different substituent in meso positions has different capacities in killing bacteria. The cationic porphyrin (4MePyP) was active against *E. coli* and *S. aureus*. The decrease in *S. aureus* survival was more than 4 log after 30 min and was almost about 5 log after 90 min against *E. coli*. The activity of $4NH_2P$ was comparable with the ionic one against *S. aureus*, while it showed no activity against *E. coli*. 4OMeP and 4MeP showed lower activity than the other porphyrins tested against *S. aureus* with a reduction in the vital count of about 2 log after 90 min, while 4PyP showeda reduction of only 1 log after 90 min. All of these three porphyrins showed no activity against *E. coli*.

Here we report the results on the full biological characterization of photoactive materials, the synthetic scaling up of the PVC-PS films, and the preliminary data collected on water disinfection processed with the use of a benchtop pilot plant.

1.1.1. PROCEDURE

Synthesis of the photoactive material

For the preparation of the photomaterial, 100 mg of PVC (high molecular weight) was taken in a 5 ml beaker and dissolved in 3 ml of tetrahydrofuran (THF). To this, 20 µl of n-octyl adipate was added and stirred till a homogeneous mixture was obtained. After, 5 mg of porphyrin were added to the suspension and stirred for 10 min. The polymer mixture was then poured onto a glass slide, spread with the help of a glass rod and then let dry in the dark up to the complete evaporation of the solvent, about 20 cm^2 PVC film was typically obtained.

To carry out tests using the benchtop, the previous procedure was adapted using 2,73 g of PVC dissolved in 82 mL of THF, 545 µl of n-octyl adipate, and 136 mg of porphyrin. The polymer mixture was poured onto a glass tray to obtain a 600 cm^2 PVC film.

Biological tests

The target microorganism that was chosen, as Gram + model, was *Staphylococcus aureus* Di4A 226. Before every test, the bacterial strain was cultured in 1 mL of Brain Heart Infusion broth (BHI) overnight at 37 °C. The culture was washed twice with Phosphate Buffer Saline (PBS), and then centrifugated for 2 minutes at 13000 rpm at 4 °C. Afterward, the obtained pellet was resuspended in 1 mL of PBS and 100 μL were put in 10 mL PBS to arrive at a bacterial concentration of 10^6 CFU/mL. Tests were carried out on a multi-well plate with 48 wells (Corning Life Science). Two wells for every condition (control and treatment in light and dark condition) were filled with 1 mL of culture. 1 cm^2 of photoactive PVC pieces were, also, added in the treatment wells.

The samples were kept under stirring and irradiated with a homemade multi-LED (130 blue LEDs TLWB7600 Vishay) with maximum emission at 470 nm, and the fluence rate regulated to 50 W/m^2 (measured by Delta OHM HD 2302.0 Light meter). Every 30 minutes, aliquots were withdrawn with a micropipette from each well for analysis. During the illumination experiments, 100 μL of the microbiological sample were taken out and were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) with Maximum Recovery Diluent (MRD). All the experiments were conducted in triplicate.

The concentration of bacteria was determined according to the Drop Plate method (Herigstad, Hamilton, & Heersink, 2001) in PCA. As can be seen in Figure 2, after serial dilutions, agar plates were divided into fourths. Each serial dilution of the sample will occupy one quadrant of each plate. 50 µl of the sample were taken out, split into 5 drops, and placed onto the quadrant of the Petri plates that have been labeled for that particular dilution of the sample.

Plates were incubated at 37 °C for 24 hours and, then, colonies grown on the agar medium were counted. Viable cell counts are expressed as colony forming units (CFU)/surface area. After every test, the photoactive PVC films have been cleaned with a cotton swab dipped in commercial denatured alcohol and stored in the freezer.

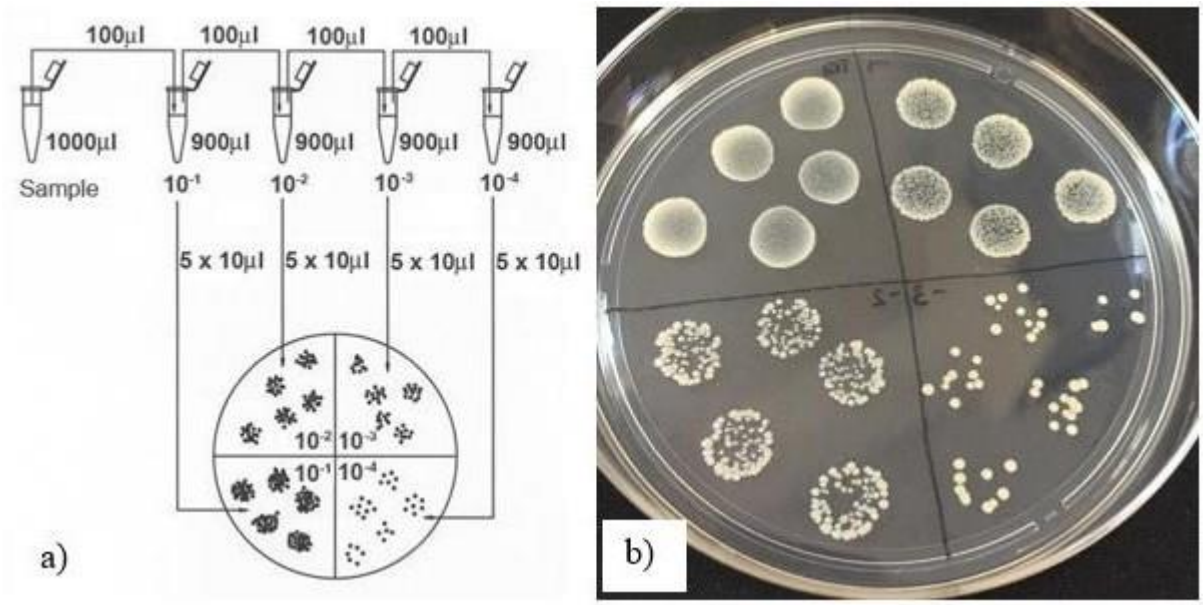


Figure 2: a) Dilution series followed by drop-plating technique, b) Example of a colonized agar plate, divided into four quadrants and containing five evenly spaced drops of diluent

Tests in benchtop

Before each use, 3 L of deionized sterile water was passed through the benchtop. *S. aureus* Di4A 226 was cultured in 10 mL of BHI overnight at 37 °C. The culture was washed twice with PBS, and then centrifugated for 2 minutes at 13000 rpm at 4 °C. Afterward, the obtained pellet was resuspended in

10 mL of PBS and then put in 1 L PBS to arrive at a bacterial concentration of 10^6 – 10^7 CFU/mL. A 560 cm^2 PVC film was placed inside the benchtop and the sample was passed through the system. The benchtop was irradiated with nine incandescent light bulbs (Philips, 24V 60W) with a fluence rate regulated to 200 W/m^2. Every 30 minutes, 100 μL of the microbiological sample were taken out and were serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) with MRD. All the experiments were conducted in duplicate. The concentration of bacteria was determined according to the Drop Plate method in PCA.

1.1.2. RESULTS

Data reported in Report 3 demonstrate an abatement of more than 3 log against *S. aureus* of a PVC film added with 5 % w/w of 5,10,15,20 – (Tetra-4-methoxyphenyl) porphyrin. Therefore, for each porphyrin, a material was synthesized and the biological characterization of all the materials was made. Figure 3 reports the survival trends of *S. aureus* treated with each material. The activity of PVC-4NH_2P and PVC-4PyP was higher than that of PVC-4OMeP and PVC-4MeP. PVC-4NH_2P and PVC-4PyP showed a reduction of about 4 log and 3 log respectively after 90 min against *S. aureus*. PVC-4MeP showed no activity while PVC-4OMeP showed a reduction of about 1 log after 90 min.

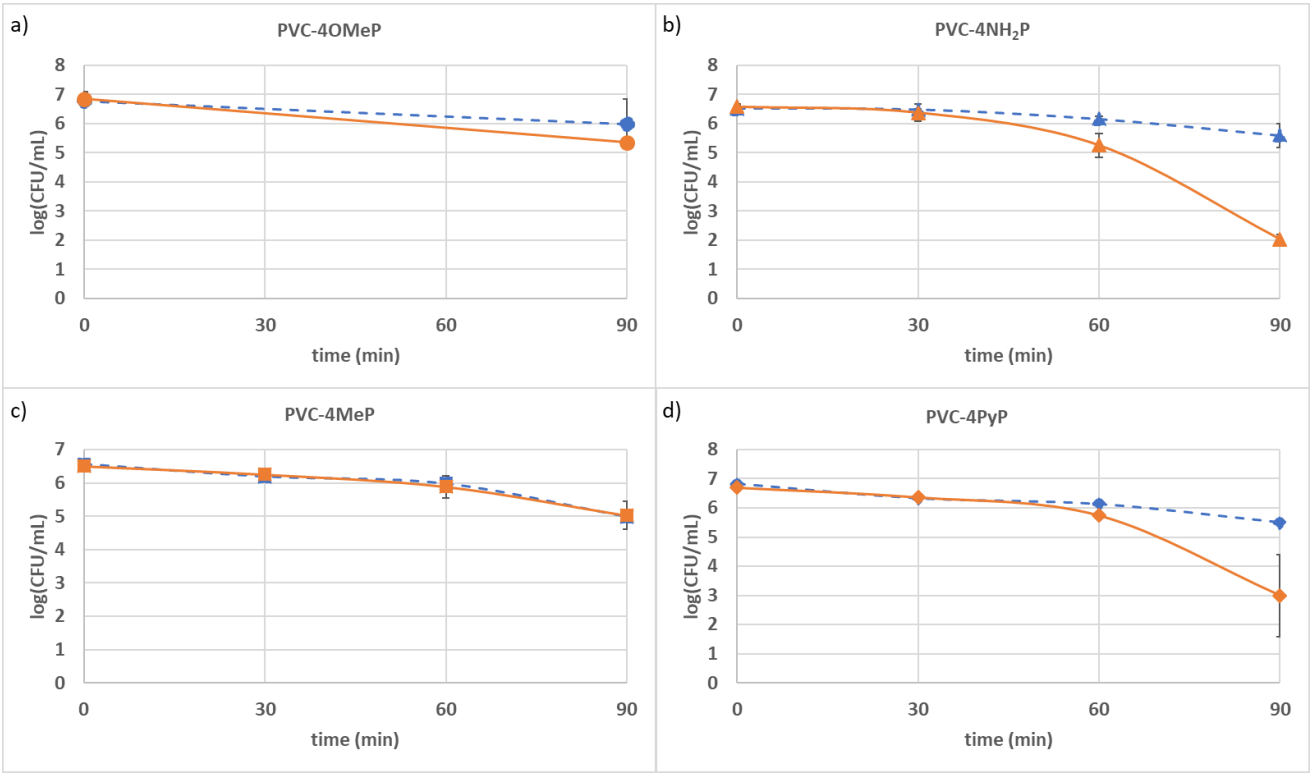


Figure 3 Photodynamic efficiency of different photo-materials vs S. aureus 226: a) PVC-4OMeP, b) PVC-4NH_2P, c) PVC-4MeP, d) PVC-4PyP. Dashed blue lines refer to the control, while the continuous lines refer to the samples irradiated with light at 50 W/m^2 fluence rate. Error bars are reported on the graph, when they were not visible they were below the dimension of the symbols.

In most cases, the photosensitizer embedded into the polymeric matrix does not retain the same efficiency shown when dissolved into the bacteria solution. In this respect, 1 cm^2 of $4NH_2P$-PVC, containing about 250 μg of photosensitizer, has a photokilling ability comparable to its 5μM solution (containing 3,37μg of photosensitizer), but the other porphyrins did not fulfill this trend. Comparing the data in Table 2 and Figure 3, 4OMeP and 4MeP had higher activity in solution against *S. aureus* than when they were immobilized, while 4PyP had higher activity when immobilized in PVC film than in solution.

Table 2 Activity of dissolved porphyrins (5μM)

time (min)	S. *aureus* 226 concentration [log(CFU/mL)]			
	4OMeP	$4NH_2P$	4MeP	4PyP
0	6,325 ± 0,202	6,086 ± 0,468	6,123 ± 0,062	6,107 ± 0,133
90	3,632 ± 0,002	1,279 ± 0	3,817 ± 0,155	5,402 ± 0,767
Logarithmic reductions	2,693	4,808	2,306	0,705

As already mentioned in Report 3, the different abilities of porphyrins in forming ROS can be related to aggregation phenomena that can change significantly by varying the dispersion medium (solution or polymeric matrix): the more aggregated are the photosensitizers, the less they can trigger the photodynamic process. PVC-$4NH_2P$ and PVC-4PyP were then selected to further investigate the potentiality of the materials as bactericidal surfaces. The possibility of using the same materials several times for disinfection purposes has been tested: a sample of freshly synthesized material was used for a photo-disinfection experiment and subsequently removed, washed, cleaned with alcohol, and reused for the next disinfection experiment conducted on an initial bacterial concentration of 10^7 CFU/ml. This procedure was repeated until a significant drop in photodynamic activity was detected. As reported in Figure 4, reused PVC-4PyP gradually loses its antibacterial activity reaching, at the fourth turnover, almost 2 log of abatement after 90 min of irradiation, while PVC-$4NH_2P$ at the fourth reuse is still able to reduce the initial load by 3 log (after 90min of irradiation).

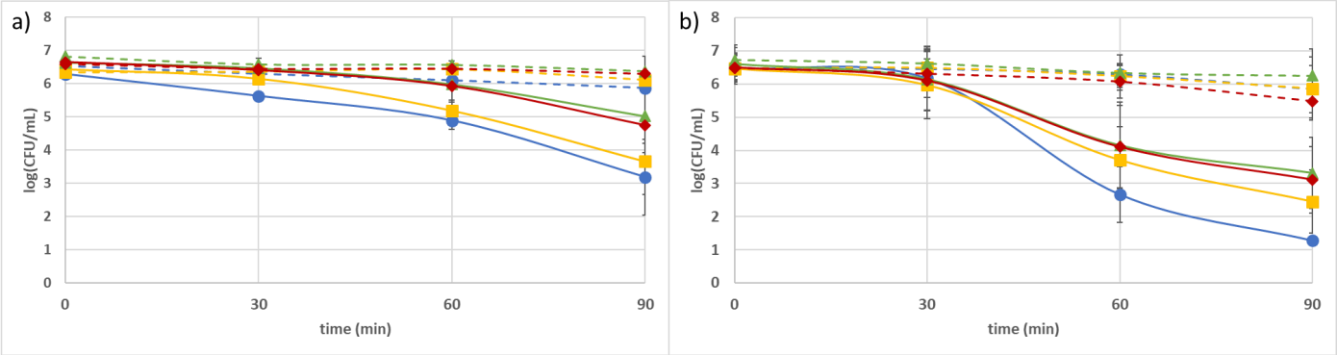


Figure 4 Photodynamic efficiency of: a) PVC-PyP, b) PVC-4NH$_2$P vs S. aureus 226, reuse up to four times. Dashed lines refer to the control, continuous lines refer to the samples irradiated with light at 50 W/m2 fluence rate. Different indicators indicate different reuse times: blue circles indicate the first use, yellow squares indicate the second use, green triangles indicate the third use, red diamonds indicate the fourth use. Error bars are reported on the graph, when they were not visible they were below the dimension of the symbols

Another important aspect to consider, given a possible transfer of this technology from the area of scientific experimentation to the application one, is the shelf life of the material. Different procedures were tempted to find that storage at -10°C allows keeping the bactericidal efficiencies of both PVC-4NH$_2$P and PVC-4PyP constant for at least six weeks (Table 3 and Table 4). On the contrary, fridge storage (4°C) seems not to be as efficient leading rapidly to unactive films.

Table 3 Data obtained testing PVC-4NH$_2$P vs S. aureus 226, over time. Data refer to the tests carried out with the material just synthesized and after 6 weeks of storage, at -10 °C. Samples were irradiated with light at 50 W/m2 fluence rate.

time (min)	**S. *aureus* 226 concentration [log(CFU/mL)]**			
	ctrl 1	**week 1**	**ctrl 6**	**week 6**
0	6,514 ± 0,167	6,574 ± 0,064	6,661 ± 0,030	6,608 ± 0,168
90	5,597 ± 0,409	2,025 ± 0,172	5,593 ± 0,859	1,290 ± 0,016

Table 4 Data obtained testing PVC-4PyP vs S. aureus 226, over time. Data refer to the tests carried out with the material just synthesized and after 6 weeks of storage, at -10 °C. Samples were irradiated with light at 50 W/m2 fluence rate.

time (min)	**S. *aureus* 226 concentration [log(CFU/mL)]**			
	ctrl 1	**week 1**	**ctrl 6**	**week 6**
0	6,682 ± 0,211	6,500 ± 0,281	6,682 ± 0,175	6,665 ± 0,160
90	5,683 ± 0,255	3,036 ± 0,927	6,115 ± 0,277	3,455 ± 0,974

Finally, to complete the characterization of the material, scanning electron microscope images were collected. The micrographs of the materials, used once and used four times, are reported in Figure 6

and Figure 7. Areas with well-organized and ordered textures together with more irregular one are visible on the material surface Figure 5.

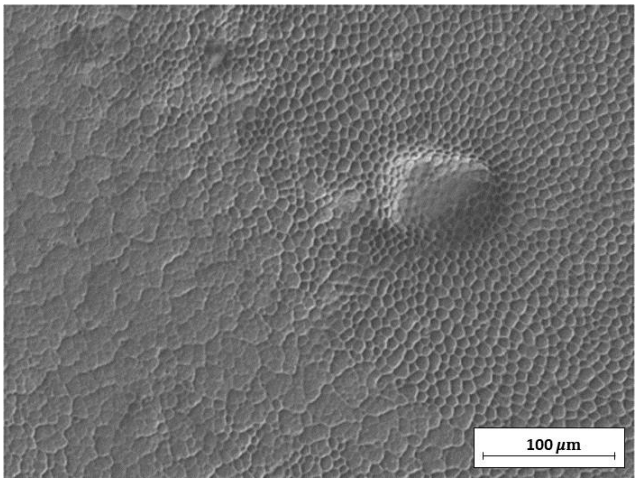


Figure 5 Scanning electron microscope (SEM) images of PVC-4PyP used once (250x magnification).

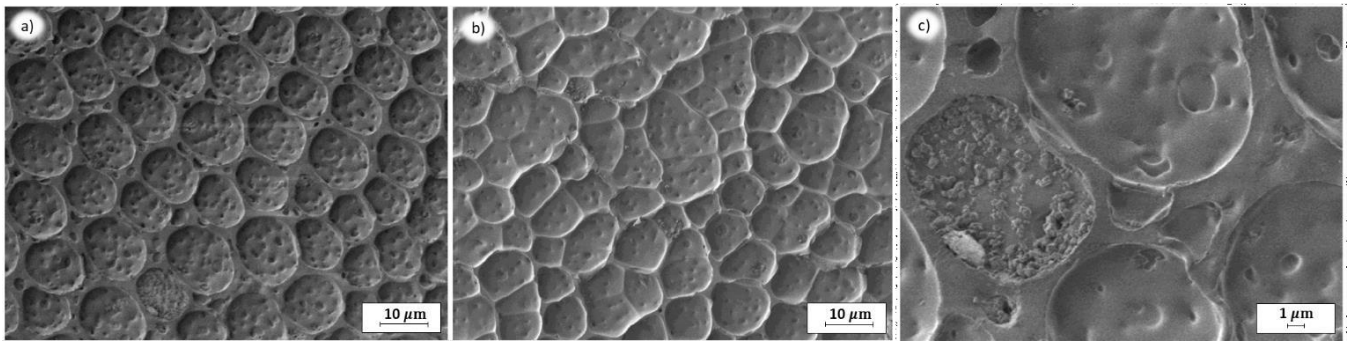


Figure 6 Scanning electron microscope (SEM) images of PVC-4PyP used once at different magnifications: a) 1300x magnification, b) 1600x magnification, c) 4500x magnification.

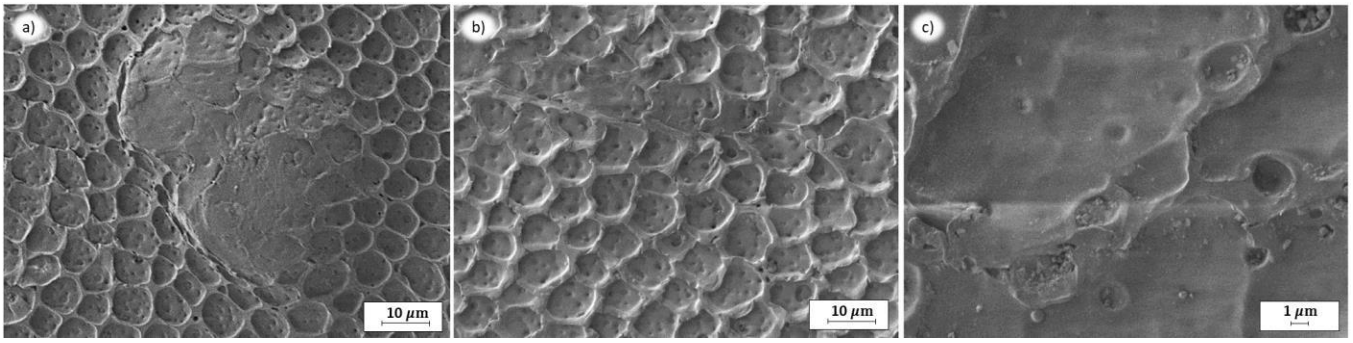


Figure 7 Scanning electron microscope (SEM) images of PVC-4PyP used four times at different magnifications: a) 1300x magnification, b) 1600x magnification, c) 4500x magnification.

The material keeps this morphology intact all over the use cycles, demonstrating that the PVC has not been decomposed by the oxidative working conditions, as the previous chemical analyzes suggested.

Finally, attempts were made to transfer this treatment technique from the laboratory scale to a larger one. To this purpose, a benchtop pilot plant designed by CAFC was used, but unfortunately, the

preliminary tests showed that the device itself caused the total death of the bacterial load within the time needed for the photodynamic treatment, this condition prevented the collection of the photo-oxidation data. All the efforts made to understand the origin of this effect and to mitigate it were in vain and therefore a new device was designed and assembled. The main differences between the two devices were in the pipes (only silicon tubes used in the second benchtop pilot plant) and in the number of metallic parts in contact with water. In this new design, the water flowed inside a sheath consisting of two A3 glasses glued with silicone and spaced 1 cm apart. A stainless steel holder allowed the insertion of the PVC films inside this space to make the bacterial solution flowing continuously on a photoactive material sheet. When starting the treatment, a set of incandescent lamps was turned on so that a fluence rate of 200 W/m^2 reached the material. One liter (1L) of a solution containing about 10^7 CFU/ml of *S. aureus* was subjected to photodynamic treatment using 500 cm^2 of PVC-4PyP. Under these conditions, after 150 min of irradiation, the initial bacterial load was reduced by 4 log (the control reduced by 1.5 log) and after 180 min of irradiation, the bacteria population was completely killed (> 6 log) while by this time the control decreased by 2 log.

2. Conclusions

In conclusion, photoactive materials showing high anti-bacterial properties and chemical stability were successfully prepared and tested on a lab scale as well on a benchtop pilot plant. Theencouraging preliminary data collected so far indicate that the technique and materials developed byUniUD could find real application for disinfection purposes of aqueous bodies.

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