BULGARIAN ACADEMY OF SCIENCES
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Department "BIOTECHNOLOGY"
Laboratory "BIOREMEDIATION AND BIOFUELS"

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ABSTRACT

On a dissertation for the award of educational and scientific degree
„ Doctor of Philosophy“

Professional direction **4.3 „Biological sciences“**

Scientific specialty **01.06.12 „Microbiology“**

on the subject:

“New photosensitizers and carbon composites as antimicrobial agents”

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The dissertation covers 148 pages, contains 56 figures and 4 tables. The bibliography includes 304 sources. The numbering of figures in the abstract does not correspond to the numbering in the full text of the dissertation.

The research related to the dissertation work is conducted in the microbiological laboratories of the Faculty of dental medicine, Medical University-Sofia, Bulgaria; Department of Microbiology at the University Medical Center Groningen, Netherlands; Diagnostic consultative Center Sofiamed, Bulgaria; Institute of Clinical Microbiology, University Clinic Münster, Germany and Laboratory "Bioremediation and Biofuels" at the Institute of Microbiology "Stefan "Angelov" - BAS, Bulgaria; Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Bulgaria.

The dissertation was discussed and directed to the defense of a meeting of the National Scientific Seminar on "Applied microbiology and microbial biotechnologies" on 16.07.2024 at the "Stefan Angelov" Institute of Microbiology - Bulgarian Academy of Sciences, "Acad. G. Bonchev" bl. 26, Sofia.

Defense materials are available in the office of the scientific secretary of the Institute of Microbiology "Stefan Angelov - Bulgarian Academy of Sciences, Acad. G. Bonchev" bl. 26, Sofia.

The defense of the thesis will take place in an open session in the presance of a scientific jury on 16.10.2024 from …… in the meeting hall of the Institute of Microbiology "Stefan "Angelov" - Bulgarian Academy of Sciences, 26 "Acad. G. Bonchev" str., Sofia.

List of abbreviations:

AC	activated carbon
ACCAg	metal-carbon composites containing silver
ACCCu	metal-carbon composites containing copper
ACCMg	metal-carbon composites containing magnesium
ATCC	American Type Culture Collection
EDTA	ethylenediaminetetraacetic acid
LED	light emitting diode
MRSA	methicillin-resistant *S. aureus*
NBIMCC	The National Bank for Industrial Microorganisms and cell cultures of Bulgaria
Pc	phthalocyanine complexes
ROS	reactive oxygen species
XRD	X-ray powder diffraction
XPS	X-ray photo-electron spectroscopy
ATP	adenosine triphosphate
BET	Brunauer – Emmett – Teller
DNA	deoxyribonucleic acid
SEM	scanning electron microscopy
PDT	photodynamic therapy

1. Introduction

Antimicrobial resistance is a global health problem, leading the World Health Organization to declare it one of the 10 greatest global threats to health. Scientific research on development of new therapeutic agents, methods and techniques with a mechanism of action different from that of widely applied antibiotics and chemotherapeutics, are gaining importance because of the fast of increasing of drug resistance.

Bacteria are in many cases the causes of diseases that spread in the area of the mouth, teeth and jaw from carious lesions, through periodontal diseases, to bacterial infections of the soft tissues.

In recent years, the available treatment methods for bacterial reduction in periodontology and cariology are very effective and can be considered as a gold standard. I order to further optimize the fight against bacterial infections in the oral cavity and their prevention and to obtain a better bacterial reduction and at the same time to minimize the negative consequences of the methods used today, this work investigates the effect of photodynamic inactivation of new metal-containing phthalocyanine photosensitizers against some of the most common microorganisms *Staphylococcus aureus, Enterecoccus faecalis, Candida albicans, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis* и *Prevotella intermedia.*

Moreover, nowadays there is a growing interest in other compounds with antibacterial potential, namely the metal nanoparticles and their oxides. Activated carbon is widely used in the purification of water and air. Metal-containing carbon composites combine properties of metals and activated carbon and thus contribute to expanding the areas of its application, based on their anti-bacterial properties. A possible application is the making of such current (as during the covid-19 pandemic) and widely used protective masks and air purifiers that are always needed in operating rooms and healthcare facilities in general. Thus, another aspect of the present work is to study the effect of inactivating of clinically important microorganisms from new metal-carbon composites.

2. Objective and tasks

The aim of this dissertation is to test the antimicrobial activity of newly synthesised metal-containing phthalocyanine photosensitizers and carbon composites, consider their application respectively as an alternative in the fight against infections in the maxillofacial region and as antibacterial air purification agents.

In connection with the objective thus set, the following tasks were identified:

2.1. Evaluation of the antimicrobial activity of the newly synthesised metal-containing phthalocyanine photosensitizers:

1. Selection of oral pathogens against which to evaluate the effect of PDT.

2. Determination of the photodynamic inactivation of reference microorganisms suspensions.

3. Determination of the photodynamic inactivation of clinical isolates suspension.

4. Testing the photodynamic inactivation of biofilms from microorganisms.

2.2. Evaluation of the antimicrobial activity of the newly synthesised metal-carbon composites:

1. Physicochemical characterization of metal-carbon composites.

2. Selection of pathogens against which to evaluate the effect of metal-carbon composites.

3. Analysis of the influence of the type of metal in the metal-carbon composites on suspensions of Gram-positive and Gram-negative microorganisms.

4. Analysis of the influence of metal concentration in the metal-carbon composites on suspensions of reference microorganisms.

3. Materials and methods

In the present study, the photodynamic inactivation of:

-*Staphylococcus aureus* 1337, (MRSA) *Enterococcus faecalis* 391574 NBIMCC, *Candida albicans* 74 NBIMCC *S. aureus* 0.5 MF with four photosensitizers: hematoporphyrin, methylene blue, zinc- and gallium-containing phthalocyanines at three irradiation intervals: 5, 12 and 20 min. With an LED lamp with a wavelength of 637 nm;

-clinical isolates of *Streptococcus sanguis, Enterococcus faecalis, Streptococcus salivarius,* with zinc- and gallium-containing phthalocyanines at two irradiation intervals: 5 and 12 min;

-*Aggregatibacter actinomycetemcomitans ATCC* 29523, *Porphyromonas gingivalis ATCC 3247, Prevotella intermedia ATCC 49046* and relevant clinical isolates with tetra-methylpyridyloxy Zn Pc; p-tetra-mercaptopyridine Zn Pc; n-tetra-mercaptopyridine Zn Pc; octa-mercaptopyridine Zn Pcat three irradiation intervals: 5, 12 and 20 min;

-MRSA 48-hour biofilms with aluminum containing phthalocyanines at irradiation interval of 5 min;

-*Enterococcus faecalis* 24-hour and 48-hour biofilm with silica phthalocyanine and 48-hour biofilm with gallium phthalocyanine as photosensitizer at an irradiation interval of 5 min was tested.

Activated carbons were synthesised by one-step hydropyrolysis, solid-phase synthesis and physical activation with CO_2. For the synthesis of metal-carbon composites, the following metals were used in an amount of 10 w/w %: magnesium (ACCMg), silver (ACCAg) and copper (ACCCu). They were added during the preparation of the activated carbon in the process of the solid-phase synthesis and after obtaining the activated carbon, by hydropyrolysis impregnation and chemical activation. The metal-carbon composites obtained by the three methods have been tested for antibacterial effect against *Escherichia coli ATCC 25922.*

Metal-carbon composites ACCCu obtained by hydropyrolysis with different w/w % of copper (2.5, 5, 7.5 and 10%) were also tested for antibacterial effect against *Escherichia coli ATCC 25922.*

Metal-carbon composites obtained by hydropyrolysis (ACCMg, ACCAg, ACCCu) were investigated for antibacterial effect against clinical isolate *S. aureus* as well.

Physicochemical properties of metal-carbon composites obtained by hydropyrolysis (ACCMg, ACCAg, ACCCu) were analyzed by scanning electron microscopy (SEM), elemental analysis, BET analysis, X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS).

The determination of the surviving microorganisms in the listed above experiments was by the standard techniques for determining the microbial count.

4. Results and discussion

4.1. Phototoxicity in microorganism suspensions from reference strains

4.1.1. Phototoxicity in microorganism suspensions from *S. aureus 1337* reference strain

In the present study, a bacterial suspension of *S. aureus* 0.5 MF was tested with four photosensitizers: hematoporphyrin, methylene blue, zinc- and gallium-containing phthalocyanines. An incubation time of 5 min was given to each photosensitizer, providing the penetration of the dye into the cells. For each photosensitizer, experiments at three exposure intervals: 5, 12, and 20 min were conducted.

Fig. 1 presents the summarised results of the experiments and the microbial count in CFU/ml was reported.

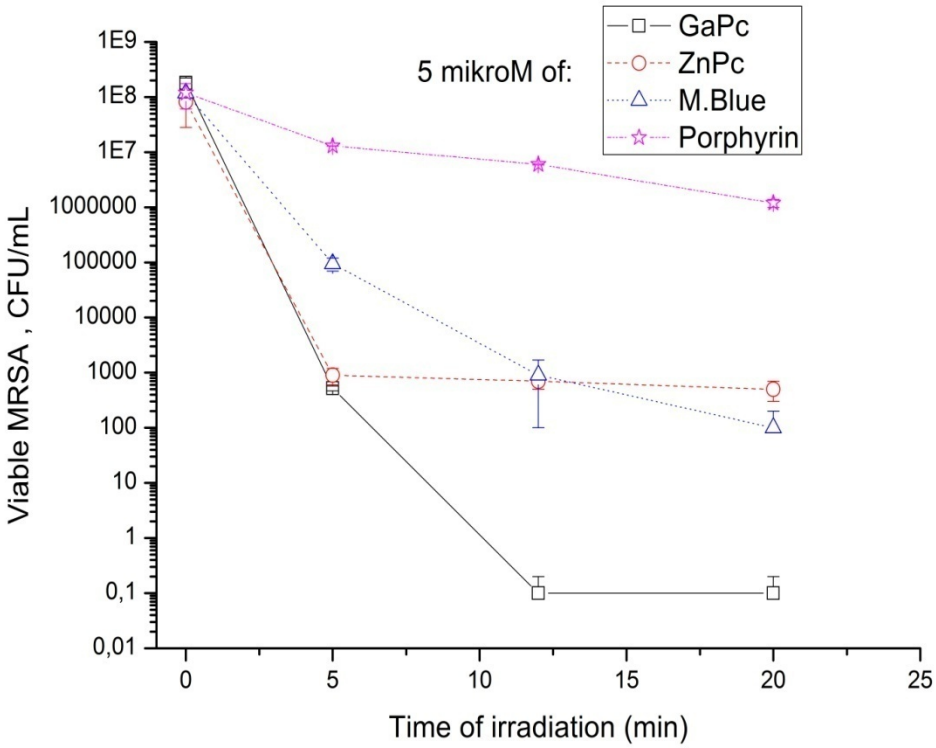


Fig. 1 Photodynamic inactivation of *S. aureus* 1337 with Ga and Zn phthalocyanine complexes (Pc), hematoporphyrin, methylene blue.

The greatest microbial reduction was achieved with gallium phthalocyanines, as exposure of 12 and 20 min led to complete destruction of bacteria, while the weakest results were observed with hematoporphyrin.

According to a study conducted to determine the effect of photodynamic therapy on 20 species of the genus *Staphylococcus* using methylene blue and a low-energy laser, the reduction of bacteria ranged from 4.89 to 6.83 (log10) CFU/ml (Miyabe et al., 2011). The present work confirms these results and suggests more effective means of inactivating *S. aureus*, which is the use of gallium phthalocyanine.

In another study, *S. aureus* suspension was exposed to LED with a wavelength of 628 nm, 14.6 mW/cm^2 and energy density of 20 J/cm^2, 40 J/cm^2, и 60 J/cm^2 in the presence of different concentrations of porphyrin (Photogem). It was worked with three concentrations of 12 µl/ml, 25 µl/ml and 50 µl/ml. The results showed that exposure to 60 J/cm^2 eliminated 100% of the bacteria. Best results in eliminating *S. aureus* were achieved by LED irradiation in combination with a photosensitizer with concentrations from 25 µl/ml to 50 µl/ml (Gois et al., 2010). In the present work, not good results were obtained using porphyrin, probably due to the lower concentrations of the substance and the lower energy density.

4.1.2. Phototoxicity in microorganism suspensions from *E. faecalis NBIMCC* 391574 reference strain

During the conducted scientific research, *E. faecalis* bacterial suspension 0.5 MF with four photosensitizers: hematoporphyrin, methylene blue, zinc- and gallium-containing phthalocyanines were tested. An incubation time of 5 min was given to each photosensitize, providing the penetration of the dye into the cells. For each photosensitizer, trials at three intervals of irradiation: 5, 12 and 20 min were conducted.

Our observations showed that the microbial number of bacterial suspension of *E. faecalis*, when treated with hematoporphyrin at 5 min decreased by 2 log, at 12 min by 3 log10, and at 20 min of irradiation by 4.5 log10. When treated with methylene blue at 5 min and 12 min of irradiation, it decreased by 2 log10, and at 20 min by 3 log10. When treated with zinc-containing phthalocyanines, the reduction was 5 log10 at 5 min, 6 log10 at 12 min, and 7 log10 at 20 min When treated with gallium-containing phthalocyanines, we report a decrease of 6 to 6.5 log10 with increasing irradiation interval from 5 to 20 min.

Fig. 2 summarises the results of the experiments. The microbial count in CFU/ml relative to the minute intervals was reported. The weakest results were observed with methylene blue and hematoporphyrin, and the greatest microbial reduction was achieved with gallium - and zinc - phthalocyanines. An exposure of 20 min resulted in full destruction of bacteria.

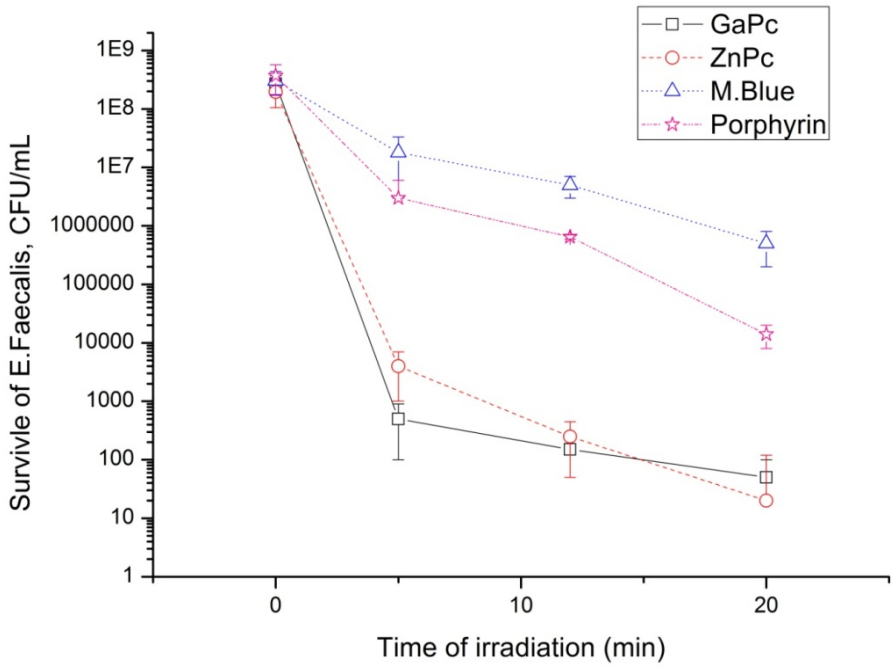


Fig. 2 Photodynamic inactivation of *E. faecalis NBIMCC 3915* with Ga and Zn phthalocyanine complexes (Pc), hematoporphyrin, methylene blue.

The graphs reflecting the decrease in microbial count in dependence on time of irradiation and photosensitizers. Concerning this object of impact, the graphs start from a common point that reflects the three controls for the four photosensitizers, due to the lack of statistically significant difference in microbial count values. For all controls the microbial count was about 10^8 CFU/ml and visibly decreased under the combined effect of the light and dye. Dependence is not directly proportional and for all four dyes is very slightly expressed, i.e. the increase in length of the irradiation interval does not lead to a significant enhancement of the effect of the photosensitizer.

4.1.3. Phototoxicity in microorganism suspensions from *C. albicans* NBIMCC 74 reference strain

Experiments continued with treatment of *C. albicans* 0.5 MF yeast suspension with four photosensitizers: hematoporphyrin, methylene blue, zinc- and gallium-containing phthalocyanines. An incubation time of 5 min was given to

each photosensitizer, providing the penetration of the dye into the cells. For each photosensitizer, experiments at three irradiation intervals: 5, 12 and 20 min were conducted. The results showed no effect on the microbial count of the *C. albicans* 0.5 MF suspension, when it was treated with hematoporphyrin. Treatment with methylene blue and gallium-containing phthalocyanines showed a reduction of the yeast 10 times only after 20 min exposure, while when treated with zinc-containing phthalocyanines with 4 log10, at 5 min of irradiation, and at 12 min and 20 min with 6 log10.

In Fig. 3 the results of the experiments are presented. On the abscissa are plotted the intervals in minutes, on the ordinate - the microbial count in CFU/ml.

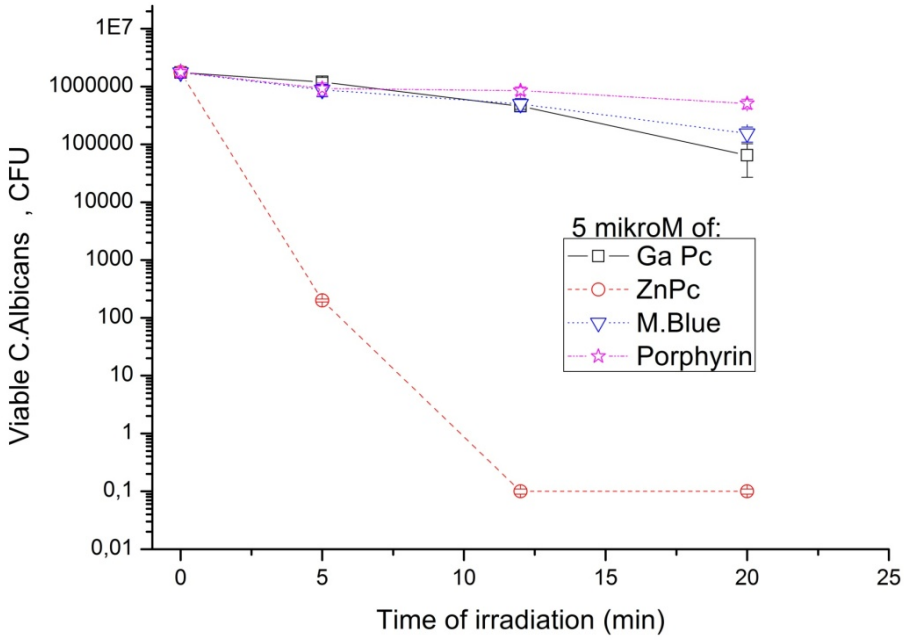


Fig. 3 Photodynamic inactivation of *C. albicans* with Ga and Zn phthalocyanine complexes (Pc), hematoporphyrin, methylene blue.

The weakest results were observed with hematoporphyrin. Very light reduction within one log10 was found with methylene blue and gallium, and the greatest microbial reduction was achieved with zinc phthalocyanines, with exposure for 12 min resulting in complete destruction of bacteria.

According to a study on LED-mediated photodynamic therapy on planktonic cultures *C. albicans* treated with erythrosine at concentrations of 0.39–200 µM and biofilms formed by *C. albicans* with 400 µM erythrosine, the effect of

PDT in planktonic cultures was 100% destruction- at concentrations greater than or equal to 3.12 µM, and regarding biofilms, a reduction of 0.74 log10 CFU/ml was reported (Costa et al., 2011).

The aim of other experiments was to influence buccal candidiasis in experimental animals. The rats treated with laser and photosensitizer methylene blue show lower levels of epithelial alteration and a weaker manifestation of chronic inflammation than the control groups (Junqueira et al., 2009). In trials with immunosuppressed mice, complete eradication of *C. albicans* from the mouth cavity using 450 – 500 µg/ml methylene blue and 664 nm diode laser wavelength was established (Teichert et al., 2002). The good results are probably due to the very high concentration of methylene blue, compared to that used in the present work, where only a tenfold reduction in microbial numbers was reached. Another study showed a dose-dependent decrease in *C. albicans* with treatment mucocutaneous and cutaneous candidiasis with PDT with photofrin. In this experiment the metabolic activity of *C. albicans* biofilm after PDT treatment was found to be lower compared to those after amphoteracin B. Both effects were carried out simultaneously (Chabrier Roselló et al., 2005).

Again, as in the previous experiments, there was no statistical significant differences in microbial count values of controls and for this reason, all the plots start from a single point, which corresponds to the three controls of four photosensitizers used. For all controls the microbial count was about 10^6 CFU/ml and it is clearly seen how it decreases at the combined effect of the light and dye. The dependance is not directly proportional and with the four dyes it is only slightly expressed, i.e. the increase in the length of the irradiation interval does not lead to substantial enhancement of photosensitizer effect, excluding the zinc-containing phthalocyanines, where the plot appears steep up to the 12th minute, when complete destruction of the yeast is achieved. In general, it can be summarized that yeasts as higher organisms were less affected by PDT.

4.1.4. Phototoxicity in microorganism suspensions from *A. actinomycetemcomitans* ATCC 29523 reference strain

In the course of experimental work, bacterial suspension of *A. actinomycetemcomitans* ATCC 29523 0.5 MF was also included with four photosensitizers water-soluble zinc phthalocyanines, with a concentration of 5 µM: (1) tetra-methylpyridyloxy Zn Pc; p-tetra-mercaptopyridine Zn Pc; n-tetra-mercaptopyridine Zn Pc; octa-mercaptopyridine Zn Pc. Controls were prepared for each experiment: light control (-/+) - no photosensitizer but illuminated; dark control (+/-) - with photosensitizer, but without light (for dark toxicity); bacterial control (-/-) - only bacterial suspension (without photosensitizer and light). An incubation time of 5 min was given to each photosensitizer, ensuring the penetration of the dye into the cells. For each photosensitizer, experiments at three irradiation intervals: 5, 12 and 20 min were conducted, with a 640 nm

wavelenght LED lamp and operating at 32 mW.cm^{-2}. The determination of the microbial count was used as the effect estimation method.

Fig. 4 summarises the results of the experiments as column chart.

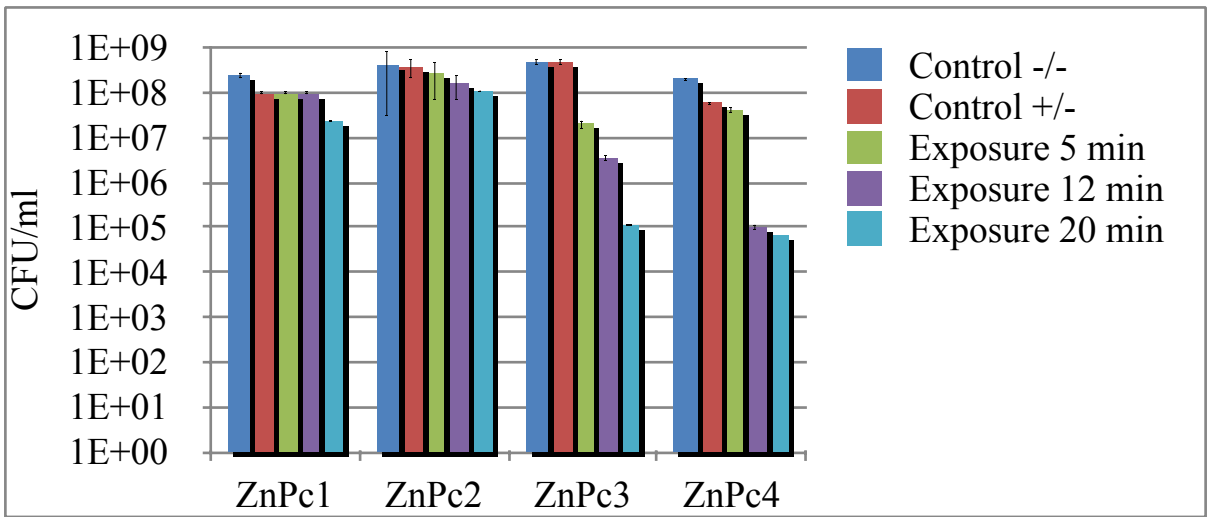


Fig. 4 Photodynamic inactivation of *A. actinomycetemcomitans* ATCC 29523 with ZnPc1 (tetra-methylpyridyloxy Zn Pc); ZnPc2 (p-tetra-mercaptopyridine Zn Pc); ZnPc3 (n-tetra-mercaptopyridine Zn Pc); ZnPc4 (octa-mercaptopyridine Zn Pc).

The four photosensitizers used are plotted on the abscissa. and for each there are 5 values of the microbial number - respectively 5 columns. The first reflects the bacterial control -/-, the second the dark control +/-, the third shows the experiment with 5 min irradiation., the fourth describes 12 min exposure, and the fifth reflects the 20 min exposure experiment. Light control is not shown due to statistically insignificant differences in microbial count values of the controls. On the ordinate the microbial count in CFU/ml is plotted.

From the diagram it is clear that with the first and second compounds there was almost no difference between the results, when irradiating the samples and controls, regardless of the exposure time. In the third compound, there is no difference between the results when irradiating the samples and controls, at 5 and 12 min exposure time, but at 20 min a reduction of 4 log10 was achieved. For the fourth compound, there is no difference between the results when irradiating the samples and controls, at 5 min exposure time, but at 12 and 20 min a reduction of 3 log10 was achieved. Strongest impact on *A. actinomycetemcomitans* exhibited an octa-mercaptopyridine Zn Pc. This result was better than the reduction achieved by the application of photosan, with an incubation time of 15 min and irradiation time of 30 and 60 min, which was one and two log10, respectively (Nerl, 2010).

In general, in Gram negative bacteria due to the complex structure of the cell wall, the penetration of the photosensitizers is difficult, especially of those

with large molecules. That is why some authors suggest using membrane-damaging agents, such as 10% EDTA in addition to photosan to achieve better results (Maisch et al., 2009). However, there are data that phthalocyanines, porphyrins and phenothiazines have an effect on both Gram positive and negative bacteria, even without such means.

4.1.5. Phototoxicity in microorganism suspensions from *P. gingivalis ATCC 3247* reference strain

Bacterial suspension *P. gingivalis* ATCC 3247 0.5 MF was also included in the scientific study and was tested with four photosensitizers, water-soluble zinc phthalocyanines at a concentration of 5 µM: tetra-methylpyridyloxy Zn Pc; p-tetra-mercaptopyridine Zn Pc; n-tetra-mercaptopyridine Zn Pc; octa-mercaptopyridine Zn Pc. Controls were prepared for each experiment: light control (-/+) - no photosensitizer, but illuminated; dark control (+/-) - with photosensitizer, but without light (for dark toxicity); bacterial control (-/-) - bacterial suspension only (no photosensitizer, no light). An incubation time of 5 min was given to each photosensitizer to ensure penetration of the dye into the cells. For each photosensitizer, experiments were performed at three irradiation intervals: 5, 12 and 20 min with an LED lamp with a wavelength of 640 nm and operating at 32 mW.cm^{-2}. Determination of the microbial count was used as a method to assess the effect.

Fig. 5 summarises the results of the experiments as column chart, similarly to the previous point.

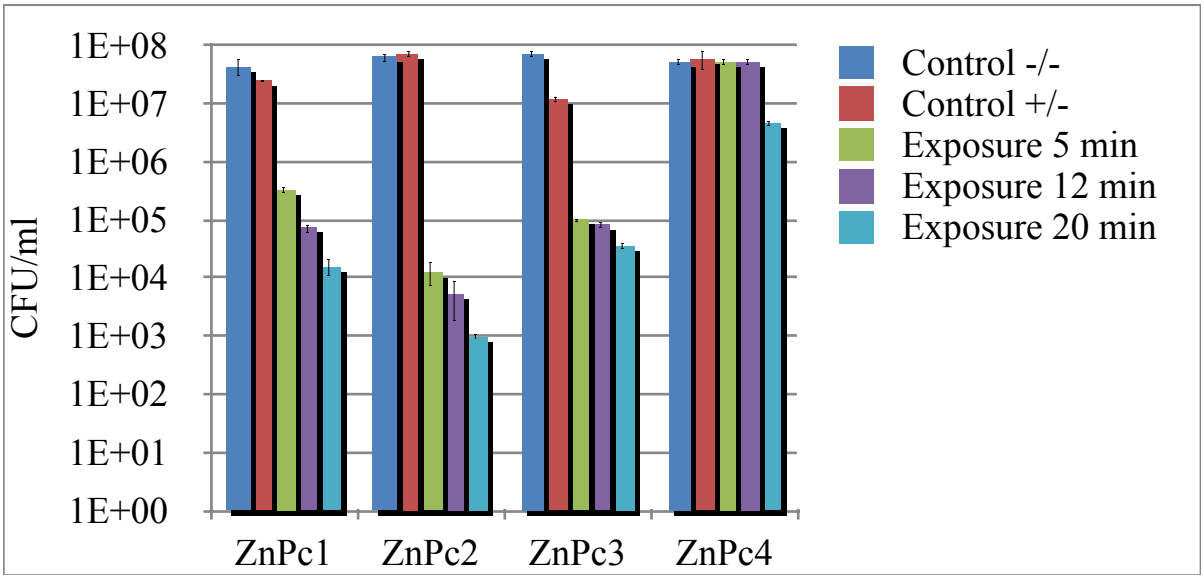


Fig. 5 Photodynamic inactivation of *P. gingivalis* ATCC 3247 with ZnPc1 (tetra-methylpyridyloxy Zn Pc); ZnPc2 (p-tetra-mercaptopyridine Zn Pc); ZnPc3 (n-tetra-mercaptopyridine Zn Pc); ZnPc4 (octa-mercaptopyridine Zn Pc).

From the diagram it is clear that in the first compound there is directly proportional dependence on the exposure time, as at 5 min a reduction of 2 lo-g10, at 12 min a reduction of 3 log10, and at 20 min irradiation a reduction of 4 log10 was achieved. At the second compound at 5 and 12 min the reduction was 4 log10, and at 20 min exposure it was 5 log10. With the third compound - at the three exposure times a reduction of about 2.5 lo-g10 was achieved. In the fourth compound, there was no difference between the results when irradiating the samples and the controls, at 5 and 12 min exposure time, but at 20 min exposure, a reduction by 1 log10 was reached. Strongest effect on *P. gingivalis* ATCC 3247 exhibited p-tetra-mercaptopyridine Zn Pc, but also with tetra-methylpyridyloxy Zn Pc a reduction of 3 log10 was reached. Octa-mercaptopyridine Zn Pc did not show good effect.

In a similar study (Rai et al., 2016), but with methylene blue 10 μM, a reduction of 3.5 log10 was achieved, upon irradiation for 20 s with a laser light with 665 nm wave length, with no linear dependence observed on the dose of ight. In an *in vivo* study investigating elimination of *P. gingivalis* also using toluidine blue, it was found that photodynamic inactivation, when applying a 630 nm diode laser was light dose independent, and depends on the concentration of the photosensitizer at lower light doses. It is believed that this effect is due to sufficient doses of energy delivered to the sample to activate all available photosensitizers, thereby generating enough oxygen radicals to kill the bacteria (Kömerik et al 2003). It has also been found that in addition to killing *P. gingivalis* cells by photodynamic inactivation, the virulence factors of this pathogen were also inactivated by the process, thus providing another advantage of this therapy over conventional treatments (Kömerik et al 2000).

4.1.6. Phototoxicity in microorganism suspensions from *P. intermedia* ATCC *49046* reference strain

An experiment followed with a bacterial suspension of *P. intermedia* ATCC 49046 0.5 MF with four photosensitizers water-soluble zinc phthalocyanines at a concentration of 5 μM: tetra-methylpyridyloxy Zn Pc; p-tetra-mercaptopyridine Zn Pc; n-tetra-mercaptopyridine Zn Pc; octa-mercaptopyridine Zn Pc. Controls were prepared for each experiment as in the previous one. The incubation time for the photosensitizers used was 5 min. For each photosensitizer, experiments were performed at three irradiation intervals: 5, 12, and 20 min with an LED lamp with a wavelength of 640 nm and operating at 32 $mW.cm^{-2}$.

Fig. 6 summarises the results of the experiments as a column chart. The number of surviving bacteria is shown, depending on the type of photosensitizer and the duration of exposure. The diagram shows that with the first compound at 5 min exposure a reduction of 6 log10 was realised. At 12 and 20 min, full inactivation of bacteria was achieved. In the case of the second compound, at 5

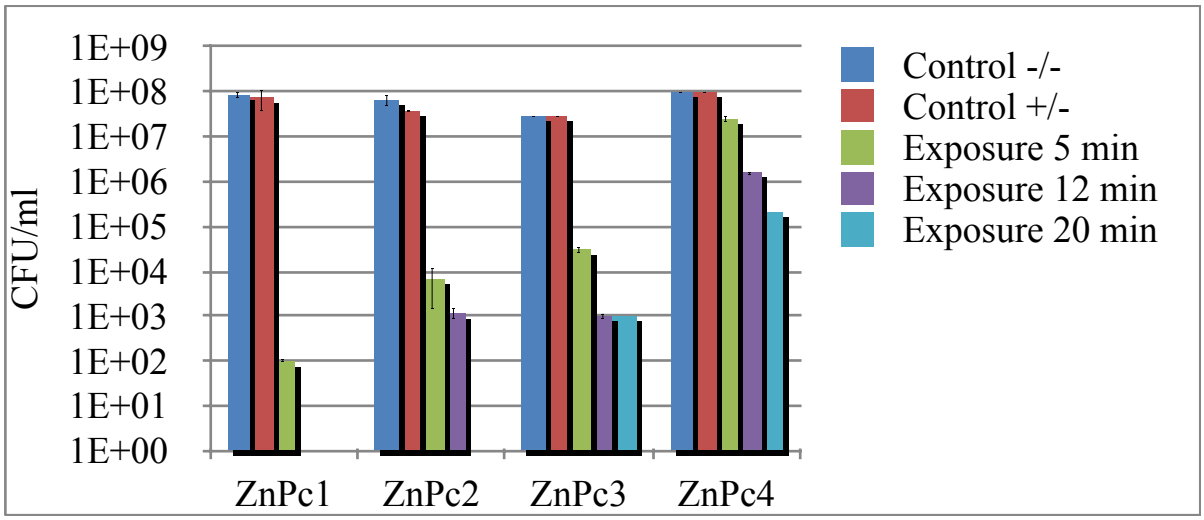


Fig. 6 Photodynamic inactivation of *P. intermedia* ATCC 49046 with ZnPc1 (tetra-methylpyridyloxy Zn Pc); ZnPc2 (p-tetra-mercaptopyridine Zn Pc); ZnPc3 (n-tetra-mercaptopyridine Zn Pc); ZnPc4 (octa-mercaptopyridine Zn Pc).

min and 12 min, a reduction of 4 log10 and 5 log10 was reached, respectively, and at 20 min irradiation a complete inactivation of bacteria was achieved. With the third compound at 5 min a reduction of about 3 log10 was achieved, and at 12 and 20 min exposure the reduction was about 5 log10. In the fourth compound there was directly proportional dependence on the exposure time, with a four-fold reduction of bacteria being reached at 5 min and at the both following exposure times of 12 min and 20 min a reduction of 1 log10 was achieved. Tetra-methylpyridyloxy Zn Pc had the strongest effect on *P. intermedia* ATCC 49046, but both p-tetra-mercaptopyridine Zn Pc and n-tetra-mercaptopyridine Zn Pc achieved a reduction of at least 3 log10, while octa-mercaptopyridine Zn Pc had no good effect.

In a similar study (Rai et al., 2016), but with methylene blue a reduction by 3log10 was achieved, upon irradiation for 20 s with 665 nm laser light. A directly proportional dependence on the dose of light was noticed as well.

P. gingivalis and *P. intermedia* are black pigmented anaerobes for which was found to accumulate endogenous porphyrins, dimeric protoporphyrin IX and monomeric protoporphyrin IX. These endogenous porphyrins have a strong absorption peak at approximately 400 nm which corresponds to the blue light. For this reason, a number of studies show that upon irradiation with blue light both pathogens can be photoinactivated in the absence of an exogenous photosensitizer (Soukos et al., 2005; Hope et al., 2013, 2016; AbdulAzeez et al., 2014). Exposure to only 665 nm laser light also resulted in significant decrease in the number of viable *P. gingivalis* and *P. intermedia*, although to a lesser extent compared to the group treated with photosensitizer (Rai et al., 2016). No such effect was observed in the present work.

4.2. Phototoxicity in microorganism suspensions from clinical strains

4.2.1. Phototoxicity in microorganism suspensions from *E. faecalis* clinical strain

A bacterial suspension of a clinical isolate of *E. faecalis* 0.5 MF was used with two photosensitizers: zinc- and gallium-containing phthalocyanines. An incubation time of 5 min was given to each photosensitizer to ensure, the penetration of the dye into the cells. For each photosensitizer, trials were conducted at two intervals of irradiation: 5 and 12 min.

Fig. 7 summarises the results of the experiments. The dependence of the bacterial reduction on exposure to the photosensitizer and the duration of irradiation is shown. The greatest microbial reduction was achieved with zinc phthalocyanines. However, none of the dye/exposure interval combinations tested resulted in complete bacterial eradication.

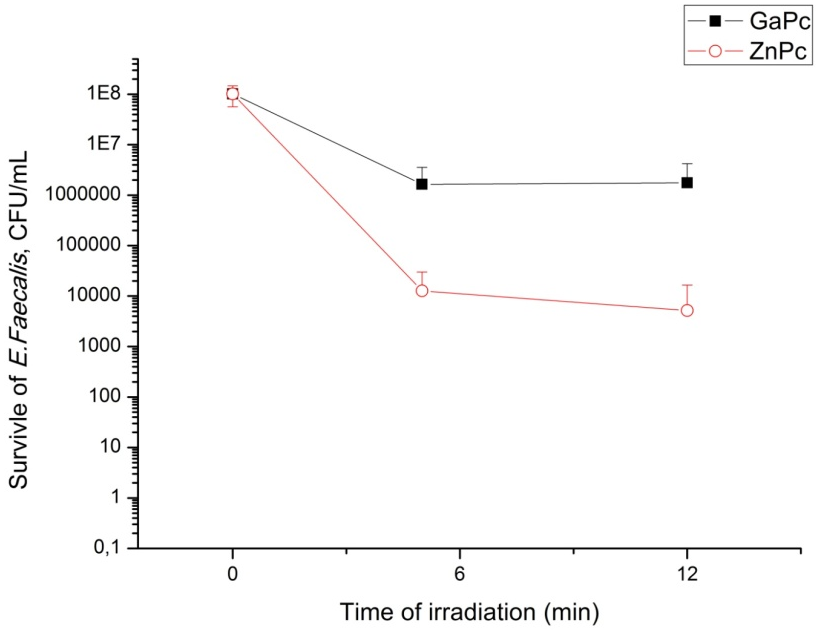


Fig. 7 Photodynamic inactivation with Ga and Zn phthalocyanine complexes (Pc) of a clinical *E. faecalis* isolate from a patient with chronic periodontitis.

The reported results showed that the microbial count of the *E. faecalis* suspension, when treated with gallium-containing phthalocyanines, decreased 100-fold at 5 and 12 min of irradiation, and when treated with zinc-containing

phthalocyanines, the decrease was 4 log10 at 5 min, and 4.5 log10 at 12 min of irradiation.

For all controls the microbial count was around 10^8 CFU/ml and it was clearly seen how it decreased with the combined exposure to the light and dye. No increase in effect was observed with increasing exposure interval for both photosensitizers, suggesting further studies with the shorter interval.

Compared to the reference strain, the clinical isolate was less susceptible to PDT.

4.2.2. Phototoxicity in microorganism suspensions from *S. salivarius* clinical strain

In the scientific experiment, a clinical isolate *S. salivarius* 0.5 MF bacterial suspension with two photosensitizers: zinc- and gallium-containing phthalocyanines was tested. An incubation time of 5 min was given to each photosensitizer. For each photosensitizer experiments were conducted at two exposure intervals: 5 and 12 min.

Fig. 8 summarises the results of the conducted experiments. The intervals in minutes are plotted on the abscissa and on the ordinate - the microbial count in CFU/ml.

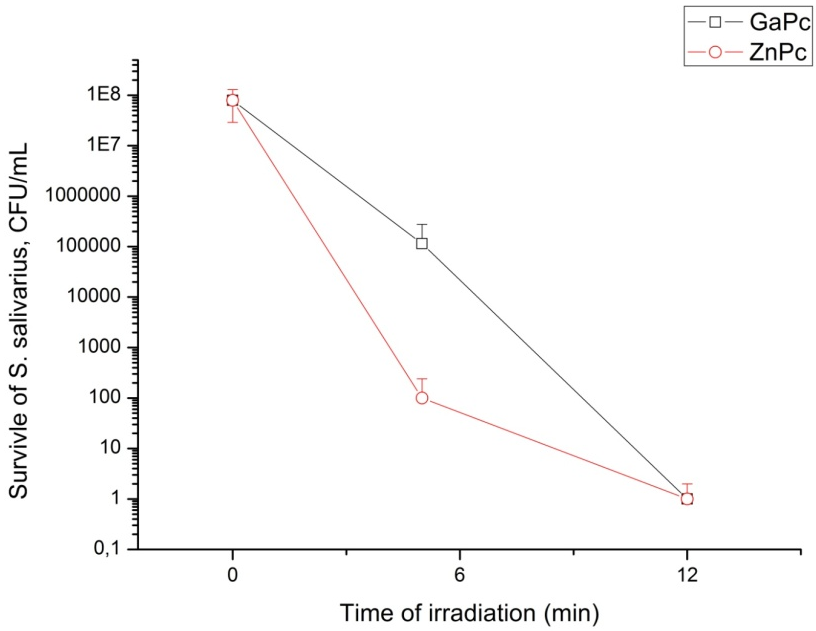


Fig. 8 Photodynamic inactivation with Ga and Zn phthalocyanine complexes (Pc) of a clinical *S. salivarius* isolate from a patient with chronic periodontitis.

The following results were observed: the microbial count of the suspension *S.salivarius* when treated with gallium-containing phthalocyanines decreased by 3 log10 at 5 and by 7 log10 at 12 min; when treated with zinc-containing phthalocyanines decreased by 6 log10, at 5 min of irradiation, and at 12 min - the reduction was by 7 log10. The greatest microbial reduction was achieved at an interval of irradiation for 12 min, both photosensitizers having the same effect. At the 5-minute interval, zinc phthalocyanine had a better effect.

An antibiogram was performed on the isolated clinical strain, which showed the following sensitivity: Clindamycin (R), Erythromycin (R), Minocycline (R), Chloramphenicol (I), Levofloxacin (S). This means that with this patient quinolones are most suitable for treatment, while macrolides, chloramphenicol and clindamycin would have no effect. Therefore, this is a good example when due to isolate resistance and sensitivity to more toxic medicationsPDT can be preferred to perform.

4.2.3. Phototoxicity in microorganism suspensions from *S. sangius II* clinical strain

In the study carried out, a bacterial suspension of clinical isolate *S. sangius* II 0.5 MF with two photosensitizers: zinc- and gallium-containing phthalocyanines. An incubation time of 5 min was given to each photosensitizer. For each photosensitizer experiments were conducted at two irradiation intervals: 5 and 12 min.

The following results were observed: the microbial count of the *S. sangius II* suspension when treated with gallium- and zinc-containing phthalocyanines decreased by 3 log10, at 5 min irradiation, and at 12 min, decreased by 3.5 log10. Better results were shown in a study of the photodynamic activation of a reference strain with methylene blue and rose bengal, in concentrations of 1.25–2.5 µg/ml and 0.62–1.25 µg/ml, respectively, when irradiated with 292 µW/cm^2 at 557 nm, 300 µW/cm^2 at 665 nm respectively. A reduction in bacteria vitality was achieved by 6 log10, while incubating for just one minute (Soria-Loz-ano et al., 2015).

The two photosensitizers had approximately the same effect, as none of the dye/exposure interval combinations tested did resulted in complete destruction of the bacteria.

Fig. 9 summarises the results of the experiments as curves describing the dependence of the surviving bacteria on the applied light dose and the photosensitizer.

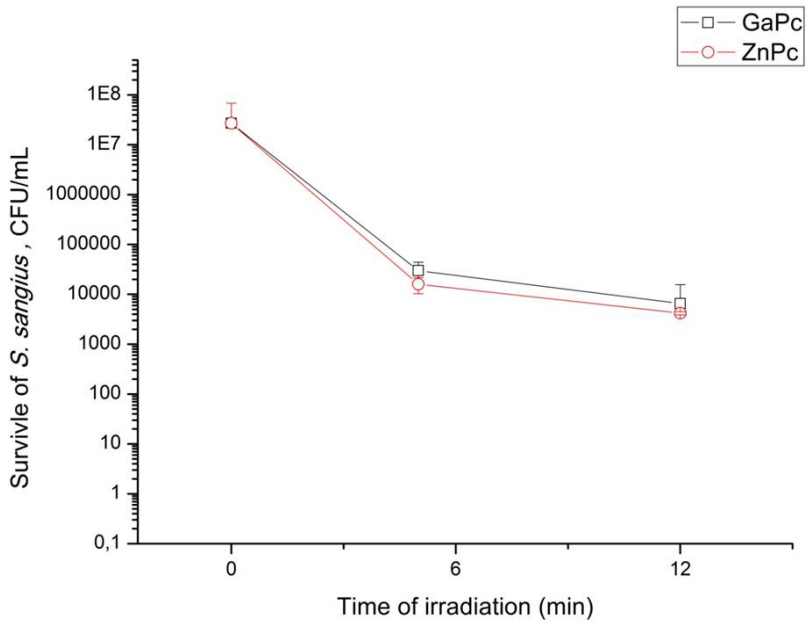


Fig. 9 Photodynamic inactivation with Ga and Zn phthalocyanine complexes (Pc) of a clinical *S. sangius II* isolate from a patient with chronic periodontitis.

Again the two plots showing the decrease in microbial count depending on the time of irradiation, start from a single point which reflects the three controls for the two photosensitizers.

An antibiogram was performed on the isolated clinical strain, which showed the following sensitivity: Clindamycin (S), Azithromycin (R), Minocycline (I), Chloramphenicol (S), Ciprofloxacin (I). This means that with this patient most suitable for treatment is clindamycin, while macrolides and quinolones would have no effect. Still the sensitivity to chloramphenicol does not make it applicable due to its high toxicity. Therefore this is another example where due to isolate resistance and sensitivity to more toxic medications can be PDT preferred.

4.2.4. Phototoxicity in microorganism suspensions from *A. actinomycetemcomitans* clinical strain

In this assay, a bacterial suspension of *A. actinomycetemcomitans* 0.5 MF was tested with four water-soluble photosensitizers zinc phthalocyanines with a concentration of 5 μM: tetra-methylpyridyloxy Zn Pc; p-tetra-mercaptopyridine Zn Pc; n-tetra-mercaptopyridine Zn Pc; octa-mercaptopyridine Zn Pc. Controls were prepared for each experiment: light control (-/+) - no photosensitizer, but illuminated; dark control (+/-) - with photosensitizer, but without light (for dark

toxicity); bacterial control (-/-) - only bacterial suspension (no photosensitizer, no light). An incubation time of 5 min was given to each photosensitizer for the dye penetration into the cells. For each photosensitizer, experiments were performed at three intervals of irradiation: 5, 12 and 20 min, with an LED lamp with a wavelength of 640 nm and operating at 32 mW.cm^{-2}. The determination of the microbial count was used as an impact assessment method.

Fig. 10 summarises the results of the experiments as column chart. It reflects the surviving bacteria after treatment with different photosensitizers at different exposure times.

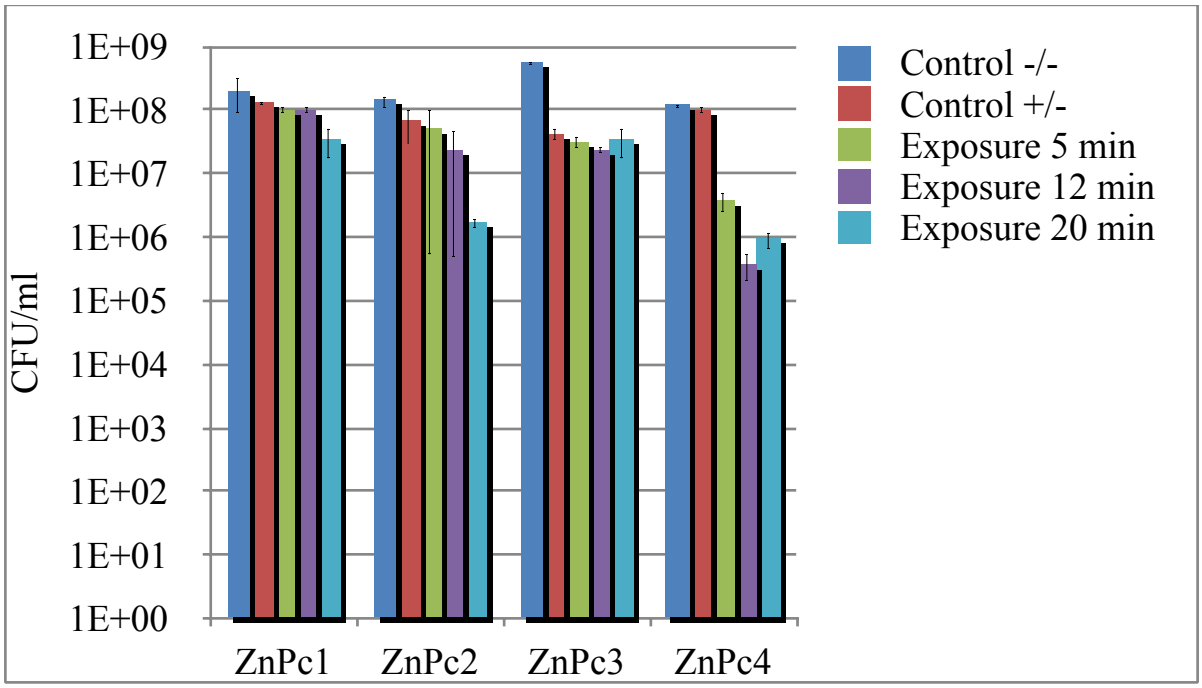


Fig. 10 Photodynamic inactivation of clinical strain *A. actinomycetemcomitans* with ZnPc1 (tetra-methylpyridyloxy Zn Pc); ZnPc2 (p-tetra-mercaptopyridine Zn Pc); ZnPc3 (n-tetra-mercaptopyridine Zn Pc); ZnPc4 (octa-mercaptopyridine Zn Pc).

From the diagram it is clear that with the first and third compounds there is no difference between the results when irradiating the samples and controls, regardless of exposure time. With the second compound, there is no difference between the results when irradiating the samples and controls, at 5 and 12 min exposure time, but at 20 min a reduction of 1.5 log10 was achieved. In the fourth compound at 5 min exposure time a reduction of about 1 log10 was achieved, and at 12 and 20 min it was achieved reduction by 2 log10. Octa-mercaptopyridine Zn Pc had the strongest effect on *A. actinomycetemcomitans*.

4.2.5. Phototoxicity in microorganism suspensions from clinical strain *P. gingivalis*

In the experimental work, a bacterial suspension of *P. gingivalis* 0.5 MF was tested with four photosensitizers water-soluble zinc phthalocyanines with a concentration of 5 µM: tetramethylpyridyloxy Zn Pc; p-tetra-mercaptopyridine Zn Pc; n-tetra-mercaptopyridine Zn Pc; octamercaptopyridine Zn Pc. Controls were prepared for each experiment: light control (-/+) - no photosensitizer but illuminated; dark control (+/-) - with photosensitizer, but without light (for dark toxicity); bacterial control (-/-) - bacterial suspension only (no photosensitizer, no light). An incubation time of 5 min was provided to each photosensitizer, for the penetration of the dye into the cells. For each photosensitizer, experiments were performed at three irradiation intervals: 5, 12 and 20 min with a LED lamp with a wavelength of 640 nm and operating at 32 $mW.cm^{-2}$. Determination of the microbial count was used as a method to assess the effect.

Fig. 11 summarizes the results of the experiments as a column chart.

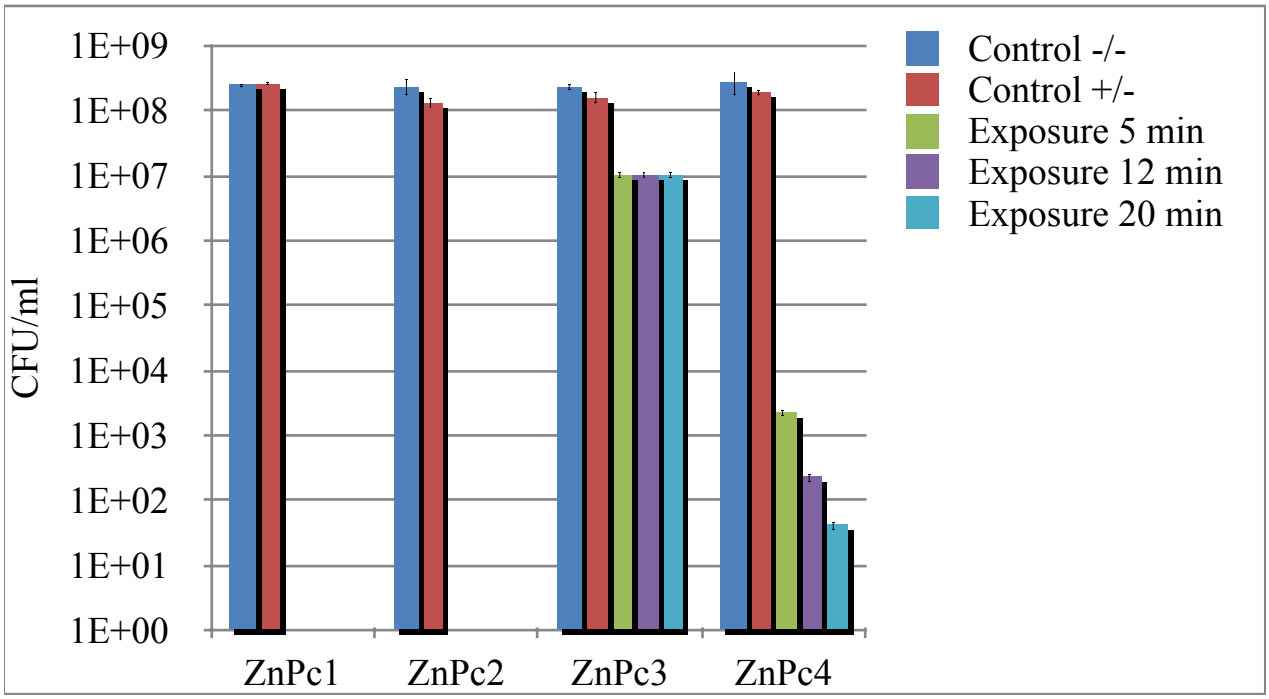


Fig. 11 Photodynamic inactivation of clinical strain *P. gingivalis* with ZnPc1 (tetra-methylpyridyloxy Zn Pc); ZnPc2 (p-tetra-mercaptopyridine Zn Pc); ZnPc3 (n-tetra-mercaptopyridine Zn Pc); ZnPc4 (octa-mercaptopyridine Zn Pc).

The diagram shows that with the first and with the second compound at 5, 12 and 20 min, complete inactivation of the bacteria was achieved. With the third compound at 5, 12 and 20 min a reduction of about 1 log10 was achieved and there was no dependence on the exposure time. With the fourth compound there was a directly proportional dependence on the time of exposure, with 5 log10 bacterial reduction achieved at 5 min and the reduction achieved at the following exposure times of 12 min and 20 min was by 1 log10 each. Tetra-methylpyridyloxy Zn Pc and p-tetra-mercaptopyridine Zn Pc had the strongest effect on *P. gingivalis*, but also with octa-mercaptopyridine Zn Pc a reduction by at least 5 log10 was achieved, while n-tetra-mercaptopyridine Zn Pc had no good effect.

An *in vitro* study analyzing photodynamic inactivation against *P. gingivalis*, used commercially available Radachlorin (0.1% Radachlorin®, InGaAlP laser device at wavelength: 662±0.1% nm, output power: 2.5 W, energy density: 6 J/cm^2, fiber diameter: 2 mm) and toluidine blue O (concentration of toluidine blue O 0.1 mg/mL, LED device at wavelength: 630 nm, output intensity: 2000 mW.cm^{-2}, tip diameter: 6.2 mm), reported a 3 log and 0.5 log decrease in survival compared to the bacterial control, respectively (Moslemi et al., 2018). Compared to experiments in this study at 10.5 J/cm^2, the bacterial reduction in the present study was greater - 3.5 log and 8 log achieved with treatment with ZnPc2 and pre-incubation for 5 minutes against the reference and clinical strain *P. gingivalis*, respectively, and with ZnPc1 - 2 log and 8 log, respectively.

4.2.6. Phototoxicity in microorganism suspensions from *P. intermedia* clinical strain

In subsequent studies, a bacterial suspension *P. intermedia* 0.5 MF was treated with four water-soluble photosensitizers zinc phthalocyanines with a concentration of 5 µM; tetra-methylpyridyloxy Zn Pc; p-tetra-mercaptopyridine Zn Pc; n-tetra-mercaptopyridine Zn Pc; octamercaptopyridine Zn Pc. Controls were prepared for each experiment: light control (-/+) - no photosensitizer but illuminated; dark control (+/-) - with photosensitizer, but without light (for dark toxicity); bacterial control (-/-) - only bacterial suspension (no photosensitizer, no light). An incubation time of 5 min was given to the applied photosensitizers, ensuring the penetration of the dye into the cells. For each photosensitizer, experiments were carried out at three irradiation intervals: 5, 12 and 20 min with a LED lamp with a wavelength of 640 nm and operating at 32 mW.cm^{-2}. Microbial count determination was the assessment method used.

Fig. 12 summarises the results of the experiments as column chart. The diagram reflects the results obtained and shows that with the first compound and with the fourth compound at 5, 12 and 20 min exposure, complete inactivation of the bacteria was achieved. With the second compound at 5 min a reduction of about 5 log10 was achieved, and at 20 min and 12 min irradiation com-

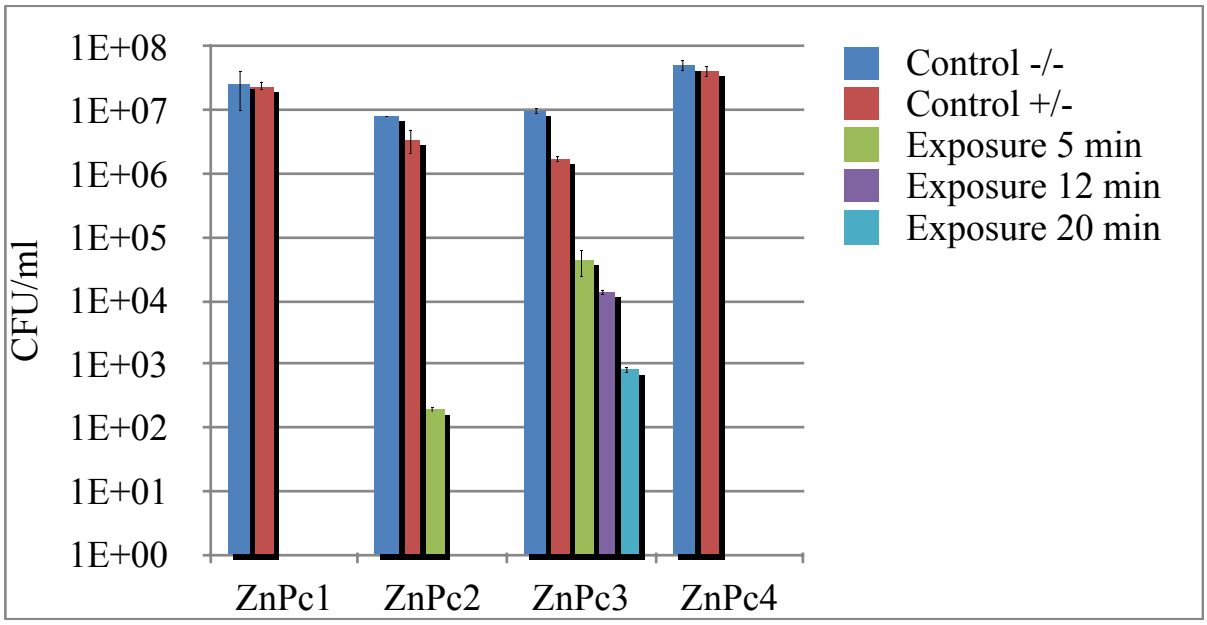


Fig. 12 Photodynamic inactivation of clinical strain *P. intermedia* with ZnPc1 (tetra-methylpyridyloxy Zn Pc); ZnPc2 (p-tetra-mercaptopyridine Zn Pc); ZnPc3 (n-tetra-mercaptopyridine Zn Pc); ZnPc4 (octa-mercaptopyridine Zn Pc).

plete inactivation of the bacteria was achieved. With the third compound, at 5 and 12 min, a reduction of about 2 log10 was realized, and at 20 min, the reduction was about 3.5 log10. Tetra-methylpyridyloxy Zn Pc and octa-mercaptopyridine Zn Pc had the strongest effect on *P. intermedia*, but complete inactivation of the bacteria was achieved with p-tetra-mercaptopyridine Zn Pc at 10 and 20 min illumination. With n-tetra-mercaptopyridine Zn Pc a reduction of about 3 log10 was achieved only at 20 min.

It is also of interest to what extent the studied photosensitizers penetrate into the target cells and how this affects their antibacterial effect. One study revealed that a high uptake into cells of reference strains *P. intermedia* and *A. actinomycetemcomitans* was observed for ZnPc 1-4, with the highest value for ZnPc3 and ZnPc4 (Kussovski et al., 2018). However, complete inactivation was recorded for *P. intermedia* after treatment with ZnPc1. The same fact was demonstrated in the present study at similar light doses. *A. actinomycetemcomitans* was less susceptible to PDT, and in contrast to the present study, where ZnPc3 was the most effective photosensitizer, ZnPc1 was reported (Kussovski et al., 2018). No strong correlation appears between uptake and effect, and for some reason different bacterial strains, within the same bacterial species, exhibit different tolerances to photodynamic therapy.

In almost all experiments including periodontopathogens, no statistically significant difference was observed between the bacterial control, dark control, and light control (data not shown), except for controls containing ZnPc3, where

a one-log reduction was observed only for clinical strains of *A. actinomycetemcomitans* and *P. intermedia* and the reference strain of *P. gingivalis*. A varying degree of effect was achieved, which appeared to depend on: 1) the chemical structure of the phthalocyanines, with p-position substituents being more effective than n-position substituents, 4-substituted compounds being more effective for *P. intermedia* and *P. gingivalis* than the 8-substituted compounds. The opposite is true for *A. actinomycetemcomitans*; 2) the dose of light, with a longer irradiation interval being more effective; 3) microbial species, with *P. intermedia* and *P. gingivalis* being more sensitive than *A. actinomycetemcomitans* and in most cases the clinical isolates of *P. intermedia* and *P. gingivalis* are even more susceptible.

In the experiments so far, bacterial suspensions have been used as test subject, but in practice the bacteria in the body are organised into biofilms, and in these structures they exhibit increased resistance to antibiotics and other damaging agents. Therefore, in order to confirm the therapeutic potential of PDT in diseases involving bacteria in dentistry, our research continued in this direction.

4.3. Phototoxicity in biofilms

4.3.1. Phototoxicity in biofilms, obtained from *MRSA*

The virulence of staphylococci is largely due to the formation of biofilms, which protects them from the action of the immune system and increases their resistance to phagocytosis and antibiotics.

The scientific study tested 48-hour MRSA biofilms with aluminum-containing phthalocyanines as a photosensitizer. An incubation time of 5 min was given, ensuring the penetration of the dye into the cells. The irradiation interval was 5 min, and as a light source a laser with a wavelength of 660 nm and an optical fiber conducting the light into the root canals were used. The following results were observed: the microbial count of the suspension obtained from the biofilm when treated with aluminum-containing phthalocyanines decreased by 2 log10 at 5 min exposure.

In Fig. 13 the obtained results are presented. The first column reflecting the -/- control is 10^5 CFU/ml. Exactly the same is the +/- control, meaning that the photosensitizer without light activation has no toxic effect. In a study on the effect of PDT for the inactivation of bacteria in biofilms, which examined the effect of toluidine blue O - cationic phenothiazine dye, on the viability and structure of MRSA biofilms, significant cell inactivation was observed when staphylococcal biofilms were co-exposed of toluidine blue O and laser. The effect was dose dependent on the radiation. Confocal laser-scanning microscopy confirmed damage to the membranes of bacterial cells subjected to PDT (Sharma et al., 2008).

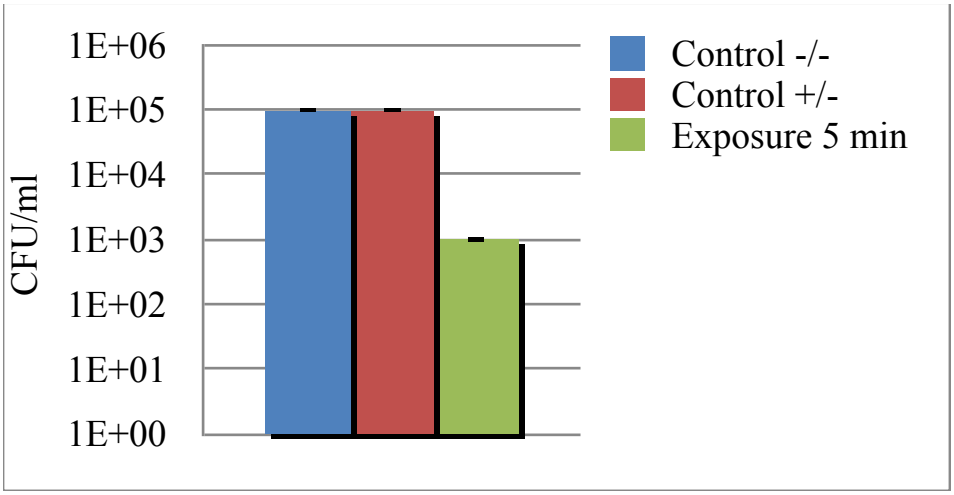


Fig. 13 Photodynamic inactivation of 48 h biofilm MRSA with Al phthalocyanine complexes (Al Pc) at 5-min exposure.

In a study comparing malachite green with phenothiazine photosensitizers (methylene blue and toluidine blue) on *S. aureus* biofilms, biofilms were subjected to PDT using a 660 nm diode laser and photosensitizer concentrations of 37.5 to 3000 µM. The best results were achieved with an average concentration of 300 µM methylene blue with a microbial reduction of 0.8–1.0 log10; 150 µM toluidine blue, with a microbial reduction of 0.9–1.0 log10; and 3000 µM malachite green with a microbial reduction of 1.6–4.0 log10 (Vilela et al., 2012). The phthalocyanine of the present work showed better results than phenothiazines and a weaker effect than malachite green.

4.3.2. Phototoxicity in biofilms, obtained from *E. faecalis*

In subsequent experiments, *E. faecalis* biofilms with Ga and Si-containing phthalocyanines as photosensitizers were also investigated. An incubation time of 5 min was given to ensure penetration of the dye into the cells. The irradiation interval was 5 min, and as a light source a laser with a wavelength of 660 nm and an optical fiber conducting the light into the root canals were used. The observed results are presented in Fig. 14. The three groups of columns reflect the results obtained for three combinations of parameters.

The first combination is an impact on a 24-hour biofilm with silicon phthalocyanine as a photosensitizer. The first column reflecting the -/- control is 10^7 CFU/ml, the second column reflecting the +/- control is 10^6 CFU/ml, so is the sample, which means that the photosensitizer without and with light activation has the same toxic effect in young biofilm.

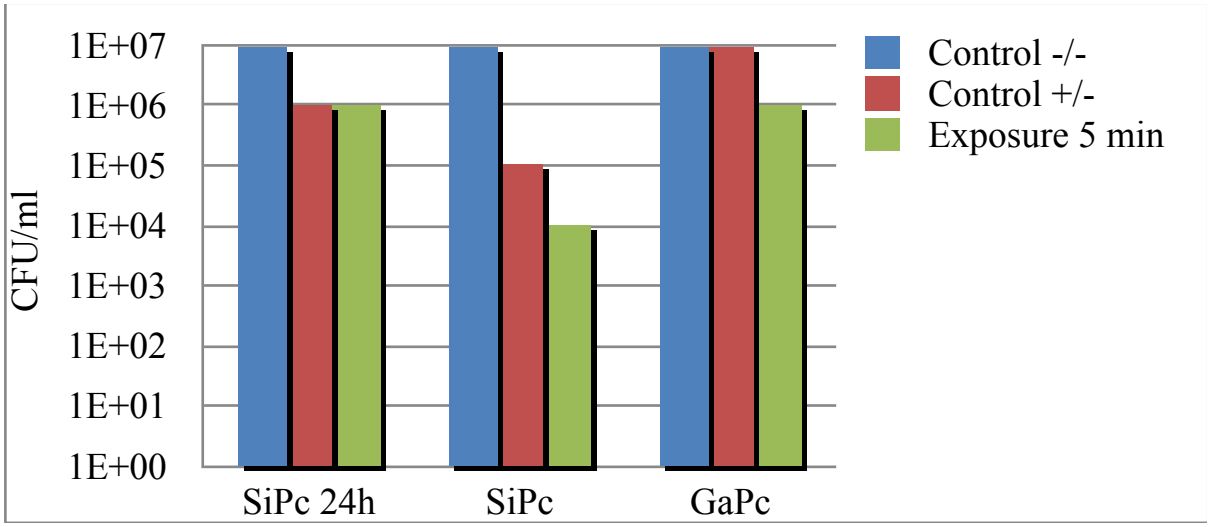


Fig. 14 Photodynamic inactivation of *E. faecalis* biofilm with Ga and Si phthalocyanine complexes (Ga Pc, Si Pc) at 5-min exposure.

The second combination is an impact on a 48-hour biofilm with silicon phthalocyanine as a photosensitizer. The first column reflecting control 1 is 10^7 CFU/ml, the second column reflecting control +/- is 10^5 CFU/ml, and the sample is about 10^4 CFU/ml, which means that the photosensitizer has a toxic effect in dark, but it is weaker from the light-activated effect.

The third combination is an impact on a 48-hour biofilm with gallium phthalocyanine as a photosensitizer. The first column reflecting the -/- control is 10^7 CFU/ml, so is the second column reflecting the +/- control, and the sample is 10^6 CFU/ml, which means that the photosensitizer has no toxic effect in the dark, and on light activation it lowers 10 times the microbial count of the biofilm.

The results of the present work are weaker than a study of photodynamic therapy using toluidine blue, laser/LED, wavelength - 660 nm, with power - 50 mW and energy used - 6.4 J, which showed 99.9% reduction of the microbial count of *E. faecalis* from a 48-hour biofilm (Fonseca et al., 2008). Another study of the effect of photodynamic therapy with methylene blue at a concentration of 6.25 µg/ml, again on a 72-hour *E. faecalis* biofilm, formed on extracted, single-rooted teeth, with a 1 W diode laser at a wavelength of 665 nm showed a 77.5% success rate in eliminating the microorganism (Foschi et al., 2007).

Comparing the effect of PDT with gallium phthalocyanine on three biological variants of *E. faecalis*, which is presented in Fig. 15, it can be seen that the effect is weaker in the clinical strain and the biofilm. This could be explained by the more complex structure of the biofilm and the presence of factors providing greater resistance to external influences in the clinical isolate.

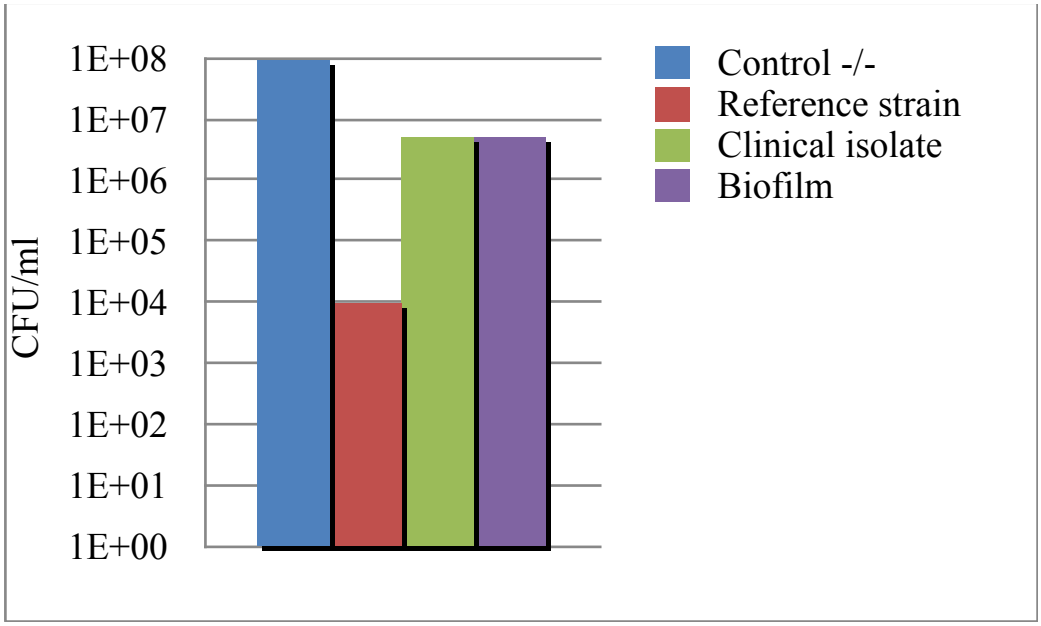


Fig. 15 Photodynamic inactivation with gallium phthalocyanine for 5 min of three variants of *E. faecalis*- reference strain, clinical isolate and biofilm.

Bacteria are a common cause of oral disease (Meyer and Fives-Taylor, 1998; Kanaki et al., 2021). The present doctoral work covers a wide range of microorganisms, the causative agents of diseases that spread in the area of the mouth, teeth and jaw: from carious lesions (*S. salivarius*), through periodontal diseases (*P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans*), endodontitis (*E. faecalis*) to bacterial infections of soft tissues (*S. aureus, C. albicans*), which are often related to each other and condition each other. In daily practice, the fight against these bacterial diseases and the elimination of their consequences represent a major task for dentists (Meyer and Fives-Taylor, 1998; Wilson, 2001; Amato, 2023).

Avoiding residual and recurrent caries, which may arise from residual microorganisms or insufficient obturation margins, is a central aspect in dental science (Pereira-Cenci et al., 2009; Thomé et al., 2009; Warreth, 2023). Above all, to obtain the most long lasting possible restorations, a bacteria-free cavity is essential (Sepetcioglu and Ataman, 1998; Duque et al., 2009; Cheng et al., 2022).

Excavation of diseased hard tooth tissue and subsequent disinfection of the resulting enamel and dentin surfaces are still the most commonly used methods to obtain a clean and germ-free cavity. Then, in daily practice, the problem of overtreatment appears, when dental instruments remove too much tooth substance, i.e. healthy tissues (Koubi and Tassery, 2008; Lavespere et al., 1996; Warreth, 2023). However, deep lesions often lead to irritation of the dental pulp and sometimes even to its opening, which again entails an additional need for treatment (Auschill et al., 2009; Koolhoven and Plasschaert, 2003; Warreth, 2023). To counter this, new ultrasound- or laser-based preparation methods are being attempted to selectively remove diseased tissue (Stiesch-Scholz and Han-

nig, 2000; Jepsen et al., 2008; Koubi and Tassery , 2008; Kornblit et al., 2009; Besegato et al., 2022). Often these approaches come with increased time, material or financial costs for both the doctor and the patient. In part of the experiments of this scientific study, a photopolymer lamp, which is available in every dental office, was used, i.e. the newly synthesised photosensitizers can be activated with light that is of the same wavelength as commonly available photopolymer lamps, and therefore no new equipment needs to be purchased.

Also, in the therapy of periodontal diseases, remaining in the periodontal pockets and in the surrounding soft tissue microorganisms are the cause of recurrent periodontopathies (Matuliene et al., 2008; Citterio et al., 2022). It follows from this that for a successful treatment, we must strive for a subsequent reduction of the pathological microorganisms in the periodontal pockets. Mechanical removal of subgingival calculus using ultrasonic devices or using curettes and scalers with subsequent disinfection by washing with substances such as chlorhexidine, which provides a long lasting effect, is a good practice (Cousido et al., 2010; Garcia-Caballero et al., 2009; Tomás et al., 2009, Brookes et al., 2020). Similar to the excavation of carious dentin, the removal of subgingival concretions goes with the loss of healthy root cementum, respectively root dentin, which can lead to weakening and eventual traumatisation of the tooth. In particularly severe cases of periodontal inflammation, systemic antibiotics are given to restore the predominantly pathological microbial spectrum to a physiological one and to reduce the number of bacteria accordingly. Disadvantages of antibiotic therapy include the occurrence of drug intolerance and side effects in the patient, as well as the development of resistance genes in bacteria (Pahkla et al., 2006; Renvert et al., 2004, 2006, 2008, 2009; Moreira and Feres - Filho, 2007; Haque et al., 2022).

Photodynamic antimicrobial chemotherapy has the potential to be an alternative to antibiotic therapy, especially for the superficial topical treatment of oral infections. The development of resistance to this method seems unlikely because singlet oxygen and free oxygen radicals interact with different cellular structures found in the microbial cell and with different metabolic pathways (Marasini et al., 2021). So, this kind of therapy is equally effective against antibiotic-sensitive and resistant bacteria, and repeated exposure does not lead to selection of resistant strains (Maisch, 2020). Superoxide dismutase and catalase protect against some oxygen radicals but not against singlet oxygen (Knopka and Goslinksi, 2007). In addition, another advantage is the avoidance of the systemic administration of drugs with all possible complications for the patient resulting from this. With PDT, the treatment is local and a possible problem can be the cessation of action when the radiation is stopped (Dai et al., 2009).

There are more than 330 publications published in the last 20 years on methylene blue-mediated PDT, which show a wide range of bacterial reduction, depending on the light source used, the dose and irradiance of the light used, and the type of pathogen (Piksa et al., 2023). For example, in this scientific

work, tetra-methylpyridyloxy Zn Pc showed photodynamic inactivation in different microbial species- *S. aureus, E. faecalis, C. albicans* and *S. salivarius, P. intermedia, P. gingivalis*, and in addition a greater reduction in the microbial count of *E. faecalis* and *C. albicans* was found compared to methylene blue (Gueorgieva et al., 2010; Angelov et al., 2011). However, the effect against *A. actinomycetemcomitans* was not good, so since a wide variety of microbial species are involved in oral infections, one approach could be to use a combination of two or more photosensitizers. One might also consider testing more strains to determine statistically what the correct dose of light and photosensitizer might be for a particular strain. As far as we know, there is no such data to date.

Indicators such as patient comfort are not to be neglected. For this reason, considering the working protocols for photosensitizers already approved for use in dentistry, those compounds that show a good effect with shorter incubation and illumination periods are more suitable (Doychinova et al., 2017; Shahbazi et al. , 2022). According to the obtained results, among the tested photosensitizers, ZnPc1 and ZnPc2 reveal the potential to meet these requirements in the treatment of chronic periodontitis.

4.4. Structural characteristics of the newly obtained composites.

Establishing the structure of the novel synthesized metal-carbon composites plays a key role in elucidating the mechanisms of impact on microorganisms and the properties of materials in general. The applied methods for characterizing the studied products and materials are among the most advanced, according to the most modern trends in scientific experimental equipment.

4.4.1. Structural-morphological analysis with scanning electron microscopy (SEM).

The SEM images of the metal carbon composites, shown in Fig. 16-18, demonstrate the presence of metal nanoparticles well embedded in the the pores of the activated carbon, which is visible in all the samples. ACCAg contains two well-differentiated types of uniform ball-like silver nanoparticles, "small balls" nanoparticles (about 1 μm in size) and „big balls” nanoparticles (about 20 μm), well distinguished on the SEM image (Fig. 16).

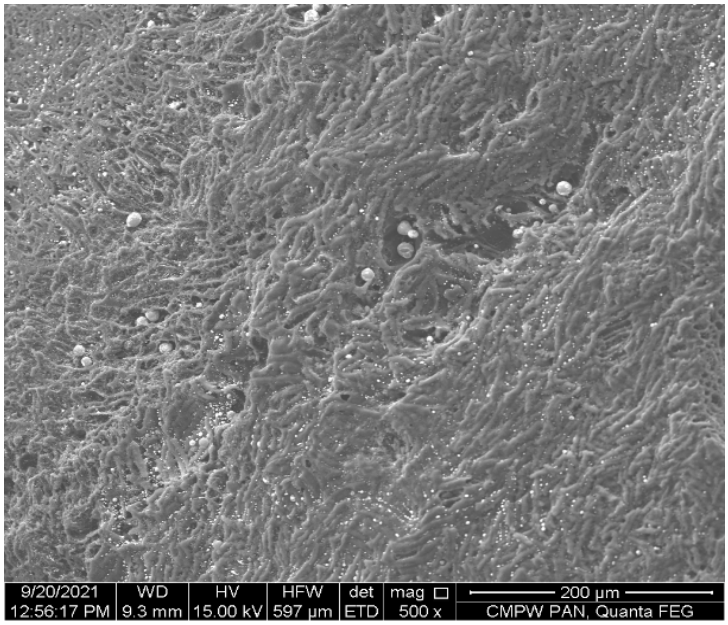


Fig. 16 SEM image of a carbon composite containing Ag.

The copper nanoparticles in ACCCu (Fig. 17) are also uniform spherical structures about 1 µm in size.

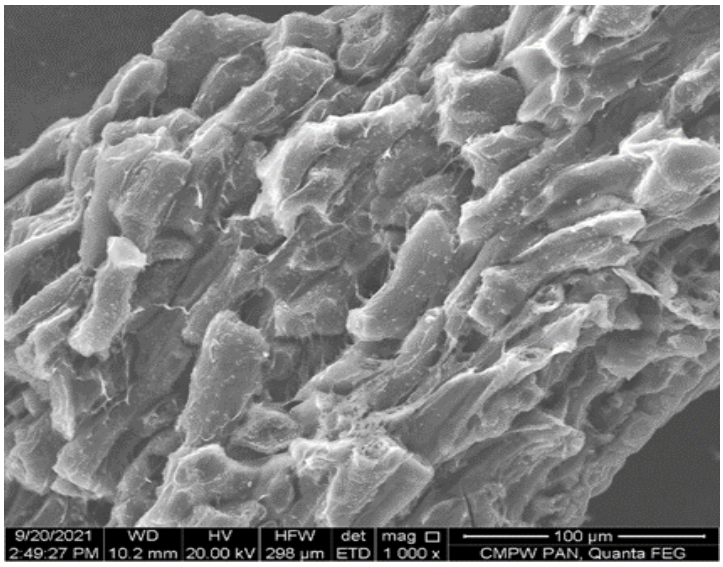


Fig. 17 SEM image of a carbon composite containing Cu.

ACCMg (Fig. 18) contains metal nanoparticles with a large variety of sizes and shapes.

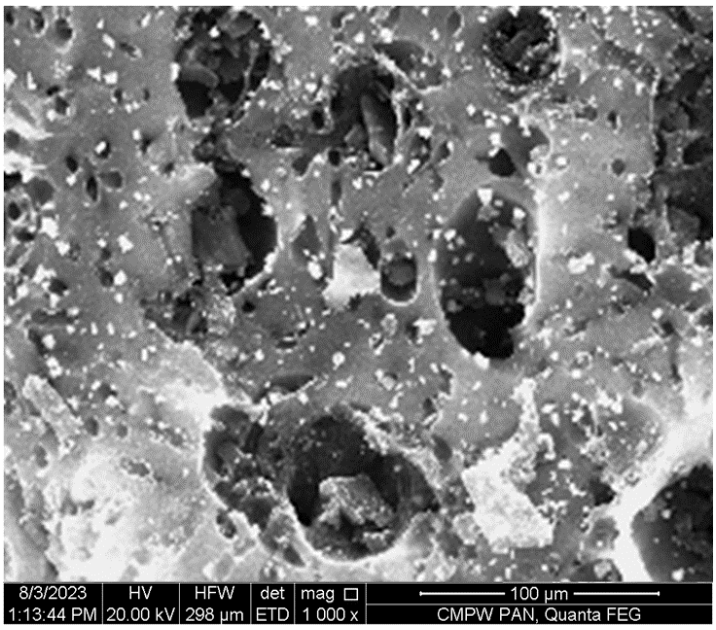


Fig. 18 SEM image of a carbon composite containing Mg.

4.4.2. XRD analysis

The XRD data (Fig. 19) show a high degree of graphitization, which is proved by the presence of highly intense narrow peaks at 24° and 44°, corresponding to the (002) and (100) reflections of 2H-hexagonal crystalline graphite, respectively. The positions of these two peaks correspond to the average values of the distances between graphite layers (or between carbon atoms in perpendicular direction to the sheets), and between carbon atoms within the layers, respectively. The position and width of the peaks are related to the mean crystal sizes. Our carbon materials are amorphous on macro scale, however on micro scale they are formed by microcrystallites that consist of tiny packets of graphite sheets. XRD results confirm the formation of metal nanoparticles on the surface of metal-carbon composites. Ag, Cu_2S, CuO and MgO were detected. In the XRD spectrum of ACCCu, Cu nanoparticles were found at $2\theta = 67°$ (Phul et al., 2018). There were also Ag-containing nanoparticles at $2\theta = 32°$ in the ACCAg sample (Mehta et al., 2016). For ACCMg, the band at $2\theta = 46°$ corresponds to MgO (Safaei-Ghomi et al., 2015).

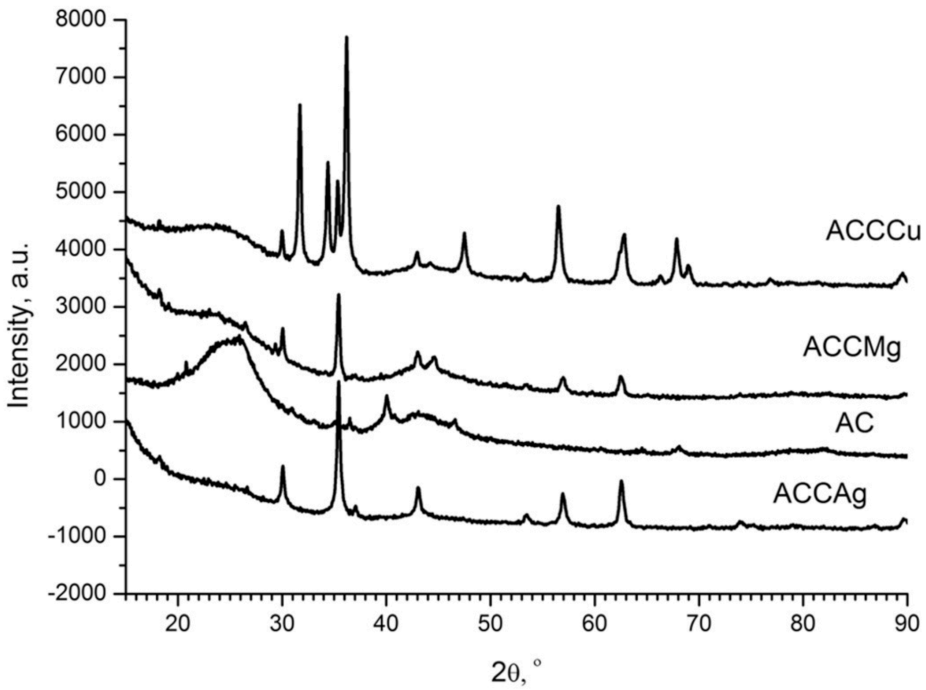


Fig. 19 XRD spectra of carbon composites and activated carbon.

4.4.3. Elemental analysis

The composites contain 73.42% carbon, 0.52% hydrogen, 0.24% nitrogen, 2.78% sulfur, 23.04% oxygen (+ losses, etc.). They are characterised by increased carbon content and decreased hydrogen content. The data obtained from the elemental analysis show a high content of sulfur, which is additionally introduced by the reagents used during the synthesis. Elevated oxygen and sulfur contents are expected to have additional antibacterial effects. The high C/H atomic ratio (11.77) indicates a relatively high degree of aromaticity and a high degree of carbonation confirmed by the XRD analysis.

4.4.4. BET analysis

The absorption isotherms of N_2 of all samples are presented in Fig. 20-23. The samples are distinguished by a large surface and the presence of micro-, meso- and macropors (Table 1).

Table 1 Texture parameters of the samples examined.

	BET surface area, m^2/g	V total, cm^3/g	V micro, cm^3/g	V meso, cm^3/g	V macro, cm^3/g
AAC	671	0.350	0.229	0.075	0.055
AACCu	595	0.296	0.194	0.054	0.048
AACMg	511	0.268	0.175	0.053	0.040
AACAg	319	0.219	0.100	0.032	0.044

All isotherms are according to IUPAC classification (Marsh и Rodriguez-Reinoso, 2006). The isotherm presents mixed type, which is hybrid between I and IV isotherm types, according to the BET classification. A hysteresis loop is clearly seen in the desorption branch at relative pressures > 0.5 – this could be attributed to capillary condensation of nitrogen in slit-shaped mesopores (Marsh и Rodriguez-Reinoso, 2006). The type and size of metal nanoparticles, as well as porous structure of carbon (established by BET investigations), determines antibacterial activity of metal−carbon composites.

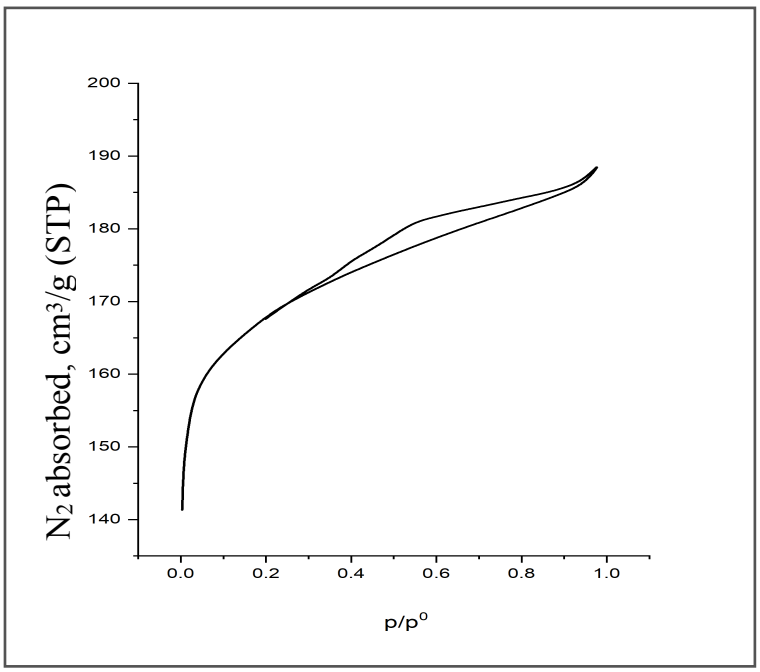


Fig. 20 N_2 adsorption isotherm of a AC at 77 K.

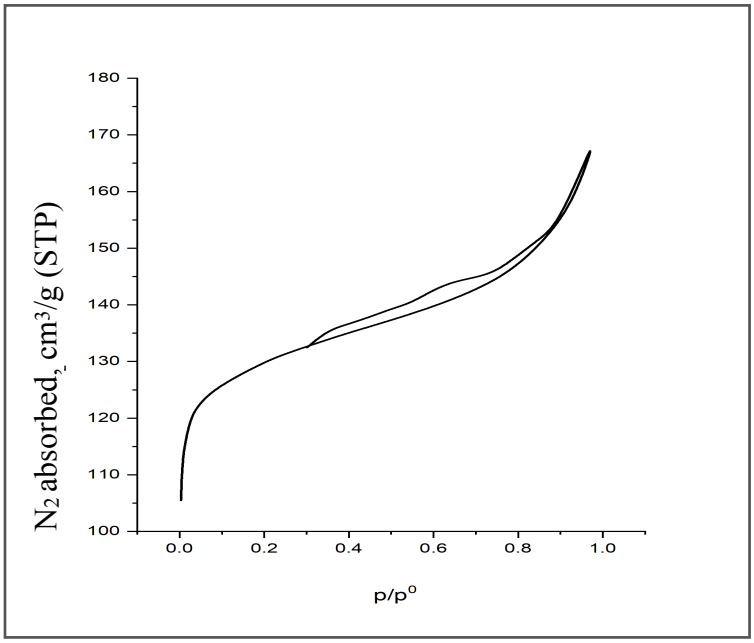


Fig. 21 N_2 adsorption isotherm of a ACCMg at 77 K.

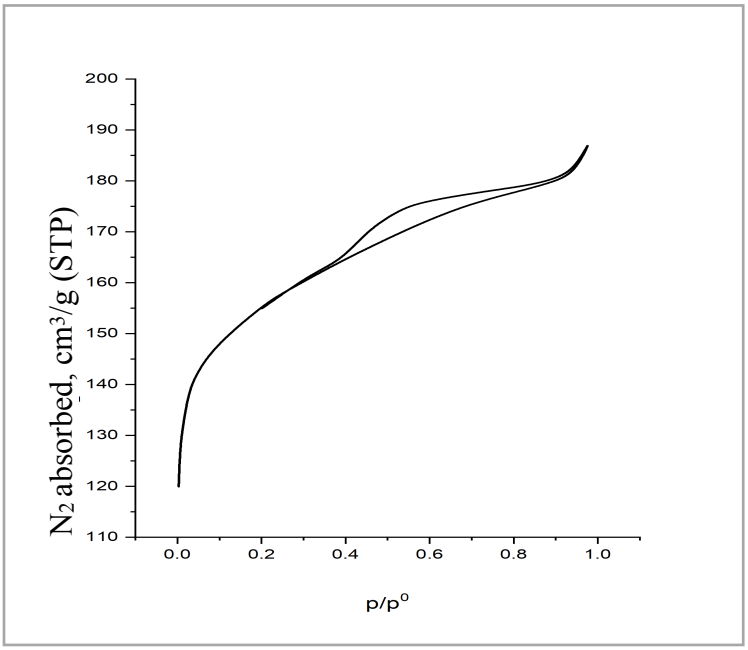


Fig. 22 N_2 adsorption isotherm of a ACCCu at 77 K.

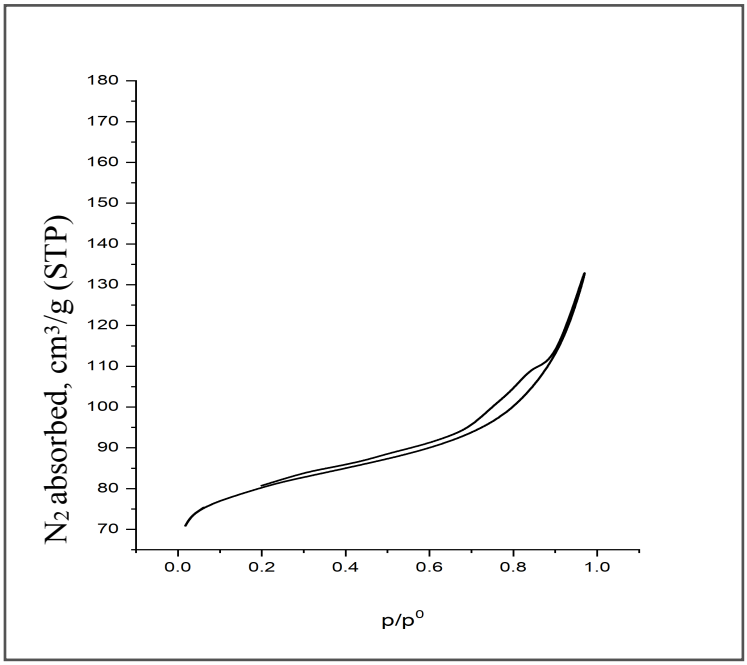


Fig. 23 N_2 adsorption isotherm of a ACCAg at 77 K.

The surface area and pore volume decrease after metal addition, due to penetration of metals, metal oxides and cations in the pores. This effect is most pronounced in the silver composite, reducing through magnesium to copper, which is in agreement with decreasing the size of their atomic and ionic radius.

4.4.5. X-ray Photo-electron Spectroscopy (XPS)

XPS data are presented in Fig. 24-26 and show the presence of Ag^0 (Fig. 24), copper in coordinations Cu^+ and Cu^{2+} (Fig. 25) in ratio 2:1, and Mg^{2+} (Fig. 26). The content of Ag, Cu and Mg, measured by XPS on the surface of the composites was 12.04 mass. %, 8.39 mass. %, and 11.20 mass. %, respectively. % за Mg.

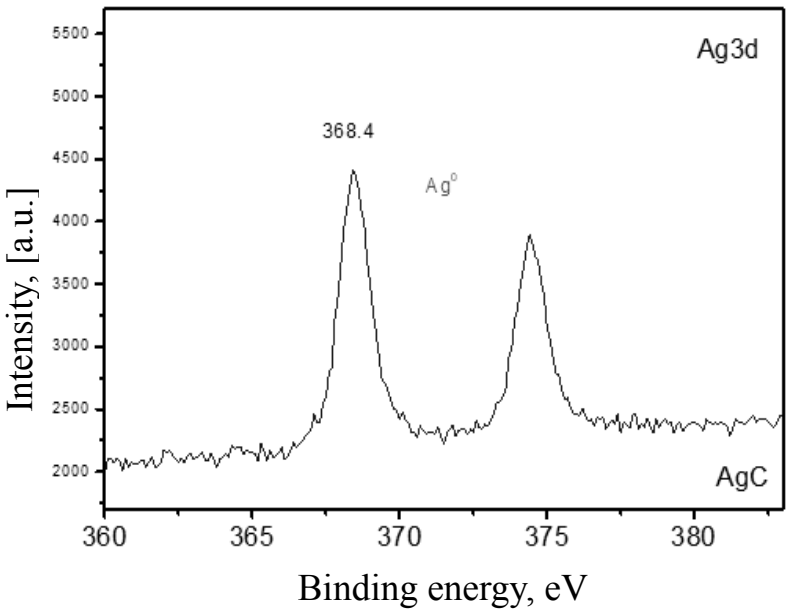


Fig. 24 XPS spectra of the ACCAg.

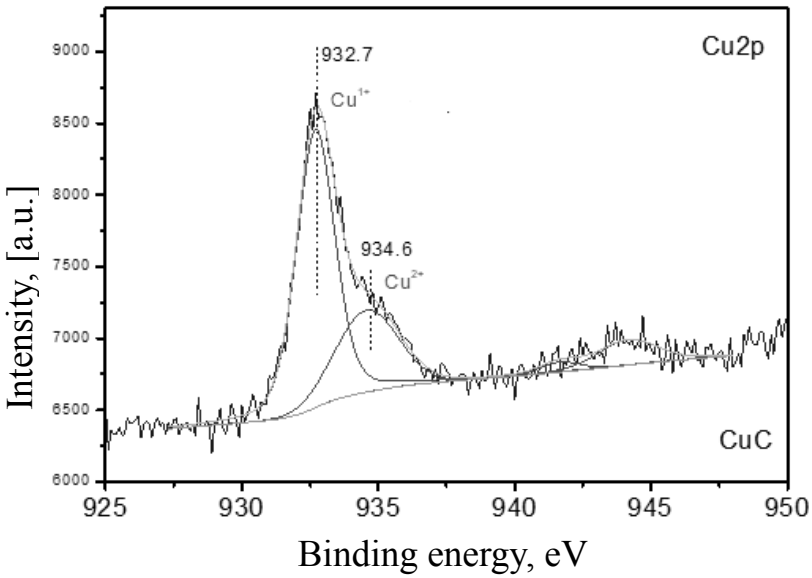


Fig. 25 XPS spectra of the ACCCu.

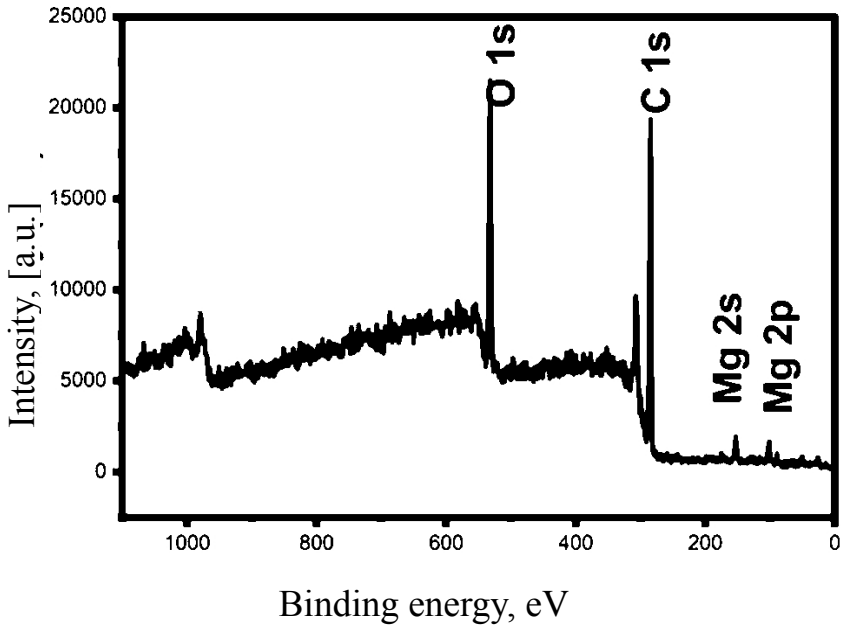


Fig. 26 XPS spectra of the ACCMg.

4.5. Antibacterial action of metal-carbon composites.

4.5.1. Testing the impact of carbon composites obtained by different methods on *E. coli* (reference strain)

Activation of carbon materials can be performed by physical and chemical methods or a combination of both (Azargohar and Dalai, 2008). Chemical activation is performed by using solid or liquid chemicals such as inorganic acids (H_3PO_4), bases (NaOH), carbonates (K_2CO_3) and salts ($ZnCl_2$); the reaction mixture is thermally treated for 2 hours in an furnace at 300-500 °C (Del Campo et al., 2015).

In addition, various gases can be used to activate carbon materials. Gaseous oxidizing agents, such as water vapor, carbon dioxide, oxygen or ozone at high temperature (600-900 °C) react with carbon by destroying aromatic structures in both amorphous and crystalline carbon. Water vapor and CO_2 are often applied because of their low cost and affordability. At lower temperatures, water vapor is preferred as it is more reactive than CO_2 at a given temperature and the molecule is smaller, resulting in better diffusion, response rate and micropores formation (Pendyal et al., 1999). Activation with water vapor leads to a product with an increased surface area and porous structure that has high sorption capacity to various adsorbates (Lima et al., 2010). Activation with CO_2 leads to more uniform pores, while water vapor activation forms a huge variety of micro-, meso- and macropores, which determines the wide application of the carbon product obtained.

Thermochemical treatment can be done also with O_2, Cl, H_2SO_4, air, etc. Oxidation by oxygen and air is used for modification of the prop- erties of carbon, and increasing oxygen-containing surface groups, playing an important role in adsorption process (Budinova et al., 1994).

The main methods of metal deposition on the support, in this case activated carbon, are ion exchange and impregnation (Bahareh et al., 2017). Ion exchange is a slow equilibrium method, so impregnation, which is faster and results in higher metal content, is the preferred embodiment in this scientific development.

In view of the above, the methods used in the present study were hydropyrolysis, solid phase synthesis and physical activation.

In our study, we chose to combine the activated carbons obtained by the three methods with nanoparticles, i.e. from each method there are three composites containing Ag, Cu and Mg. The newly synthesised composites were tested against a reference strain *E. coli*. A solution containing only the bacterial strain and a bacterial solution with activated carbon were used as the first and second controls to ensure that the antimicrobial effect was exclusively due to the composites. The samples were taken at different exposure times - 0, 1, 24 and 48 hours. The obtained metal-carbon composites were subjected to antimicrobial analysis.

As a result of the different preparation methods applied, we found the following results for antimicrobial action, presented in Fig. 27-29.

Figure 27 is a histogram representing the bacterial concentration of *E. coli* (CFU/ml) as a function of contact time (0, 1, 24 and 48 hours) with composites synthesised by hydropyrolysis. The microbial count of the first control remained in the range of 0.8-1.7x10^6, indicating that cell death did not occur naturally and the bacteria were most likely in stationary phase during the testing period. Re-

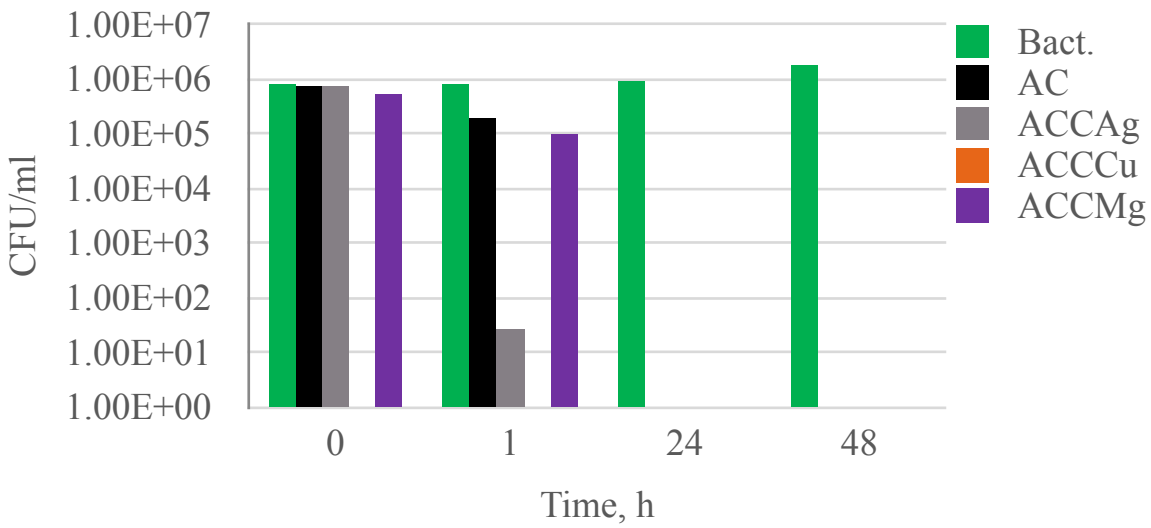


Fig. 27 Antibacterial effect of ACCAg, ACCCu and ACCMg prepared by hydropyrolysis against *E. coli* ATCC 25922.

garding the second control after 1 hour of exposure, the microbial count decreased about 10-fold and on the first and second day no viable bacteria were found in the solution. This can be explained by the fact that the very characteristics that make activated carbon such an effective filter also make it hospitable to bacteria. Bacteria or viruses easily adhere to the pores of the activated carbon, which traps them but does not kill them. Regarding the latter, some studies have shown that the pores of activated carbon used for drinking water treatment are heavily occupied by microbes, while other researchers have revealed that bacteria attached to activated carbon can be resistant to chlorination (Sommer et al., 1999; Gagnon et al., 2005), due to the formation of biofilms. When bacteria colonise the surface of activated carbon, they produce a slimy biofilm—an aggregation of microorganisms and extracellular proteins, DNA, and sugars secreted by the cells—that covers the carbon material (Trogolo, 2011). Thus we assume that as time progresses more bacteria leave the solution and harbor the AC particles and their adhesion to the composites is a reason for the resulting bacterial reduction. This hypothesis should be further approved for example with death/alive staining and confocal fluorescent microscopy.

The percentage reduction (% R) of *E. coli* at different contact time was determined in relation to the bacterial control at T_x contact time, respectively. Data are shown in Table 2.

Table 2 Percentage reduction (% R) of *E. coli* at different metal composites (Ag, Cu и Mg) and at different contact time.

Sample	%R (t=0h)	%R (t=1h)	%R (t=24h)	%R (t=48h)
AC	7.4	77.5	100	100
ACCAg	9.9	99.9	100	100
ACCCu	100	100	100	100
ACCMg	35.6	87.5	100	100

Best antibacterial effect against *E. coli* was observed when 10 % ACCCu was used with 100 % reduction of microbial count at the starting point. ACCAg and ACCMg and activated carbon only, showed the same effect at 24 h. The results indicate that the antibacterial activity depends on the contact time and the nature of the metal. Our results are similar and support the results from other studies, where to activated carbon, Ag (Yoon et al., 2008; Joshi et al., 2022), Cu (Chanthee et al., 2022; Mahlangu et al., 2022) and Mg (Tahir et al., 2023) were added. It is not quite possible to compare the data exactly since disk diffusion method has been applied by some authors, which differs from our method. Nevertheless, the same trend is established.

Figure 28 is a histogram representing the bacterial concentration of *E. coli* (CFU/ml) as a function of contact time (0, 1, 24 and 48 hours) with composites synthesised by solid phase synthesis.

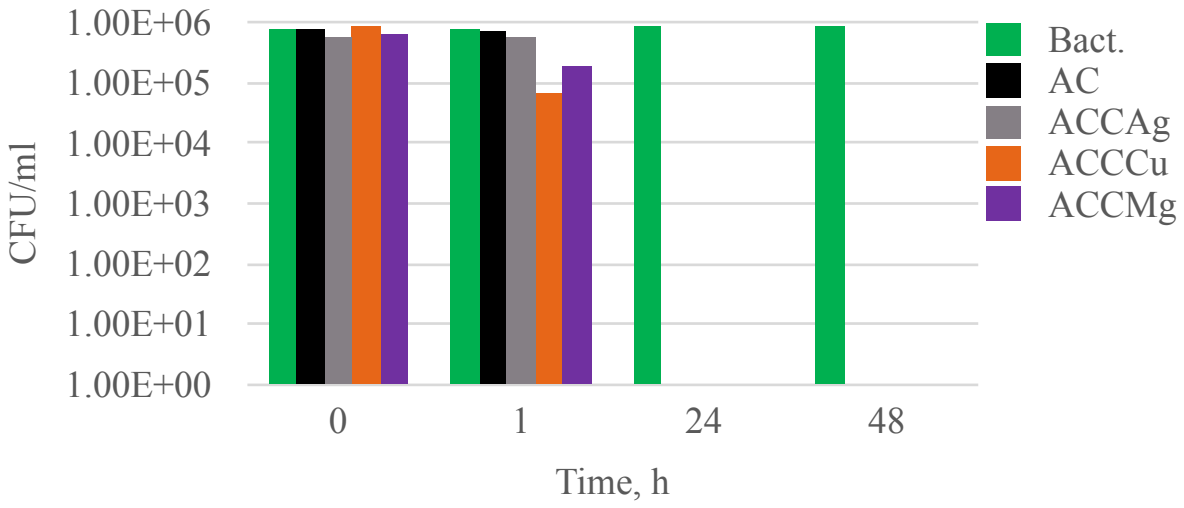


Fig. 28 Antibacterial effect of ACCAg, ACCCu and ACCMg prepared by solid-phase synthesis against *E. coli* ATCC 25922.

The solid-phase synthesis was carried out using a precursor-polymeric polyvinyl cellulose waste. This process of solid phase synthesis allows the introduction of heteroatoms (atoms other than C - S, N, O, metals) into the structure and volume of carbon composites, while, for example, in impregnation, ion exchange, spray pyrolysis and other methods, metal particles and ions are mainly retained on the surface.

The best antibacterial effect against *E. coli* was observed again when 10% ACCCu was used, but the microbial count reduction was only 10% after 1 hour. After 24 hours, no bacterial growth was detected on the cultures of the samples, except for the bacterial control. The results show that the addition of metal nanoparticles inside the composite does not contribute to increasing the antibacterial effect of the material. Probably, the interaction with microorganisms takes place most actively at the boundary surface.

The third method uses physical activation with CO_2. Physical activation produces a carbon material with uniform pore size, in contrast to hydropyrolysis, which results in the formation of a wide range of pores.

Figure 29 is a histogram representing the bacterial concentration of *E. coli* (CFU/ml) as a function of contact time (0, 1, 24 and 48 hours) with composites synthesised by physical activation.

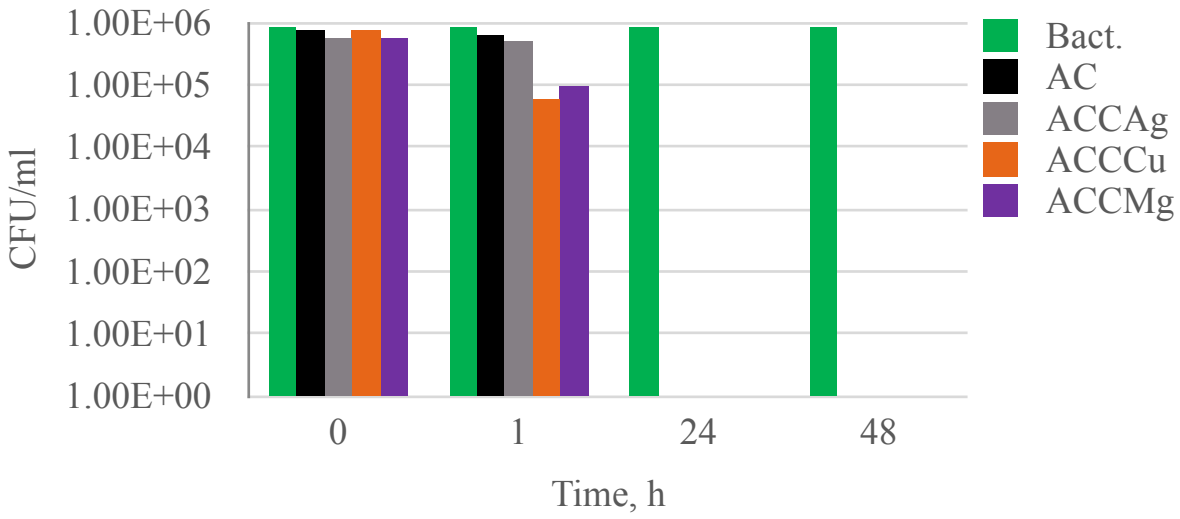


Fig. 29 Antibacterial effect of ACCAg, ACCCu and ACCMg prepared by physical activation against *E. coli* ATCC 25922.

As a result of the various applied methods, we found close values for their bacterial impact, which is most noticeable for ACC containing copper around 10 wt. % (proved by XPS above) obtained by the one-step hydropyrolysis. This method is an original method for the pyrolysis of various precursors - coal waste, raw materials of vegetable origin, polymers, etc., and was developed in the "Chemistry of Solid Fuels" laboratory at the Institute of Organic Chemistry of the BAS. It produces a carbon adsorbent with a highly developed porous structure and high adsorption capacity. The new method for obtaining carbon adsorbents is one-stage, i.e. combines the processes of carbonisation and activation with steam and takes place at relatively low temperatures (500-600 °C), which makes it economically much more profitable. Also, the use of waste as raw material, besides reducing the cost of the final product, also has an environmental aspect, i.e. can be considered a green technology.

Hydropyrolysis leads to the formation of a carbonaceous material containing a large amount (in number and volume) of pores in a wide range - micropores (< 2 nm), mesopores (2–50 nm) and macropores (>50 nm). The presence of pores on the surface of the nanoporous carbon material determines its excellent adsorption properties towards various sized atoms, molecules, ions, bacteria, viruses, etc., in gas and liquid media. The localization, coordination and properties of these metal particles and ions is the key to their antibacterial activity.

4.5.2. Effect of carbon composites obtained by hydropyrolysis on suspensions of *E. coli* reference strain

In order to determine the best method for further study of the antibacterial activity of the obtained carbon composites, we chose the following main criteria: the greatest antimicrobial activity of the composites, which includes the reduction of the microbial number and the exposure time; most optimal method for obtaining the composites, which is determined based on time consumed, labor intensity, energy intensity, price of raw materials; and quality of the obtained composites - specific surface area, uniformity of pores, distribution of nanoparticles in the matrix. Based on the analysis carried out so far, hydropyrolysis is the best method, which completely meets the requirements set in this way, which is why the composites obtained by this method are used in the subsequent experiments.

To evaluate the impact of metal concentration on the antibacterial effect, we proceeded with further testing of the material that showed the best result, namely ACCCu. Sampling times, controls and bacterial suspension were the same as in the previously discussed investigation except ACCs containing 2.5%, 5%, 7.5% and 10% copper were added to the bacterial solution.

Figure 30 presents the bacterial survival of *E. coli* (CFU/ml) as a function of contact time and metal concentration. The longer the contact time and the higher the copper content, the lower the microbial count obtained.

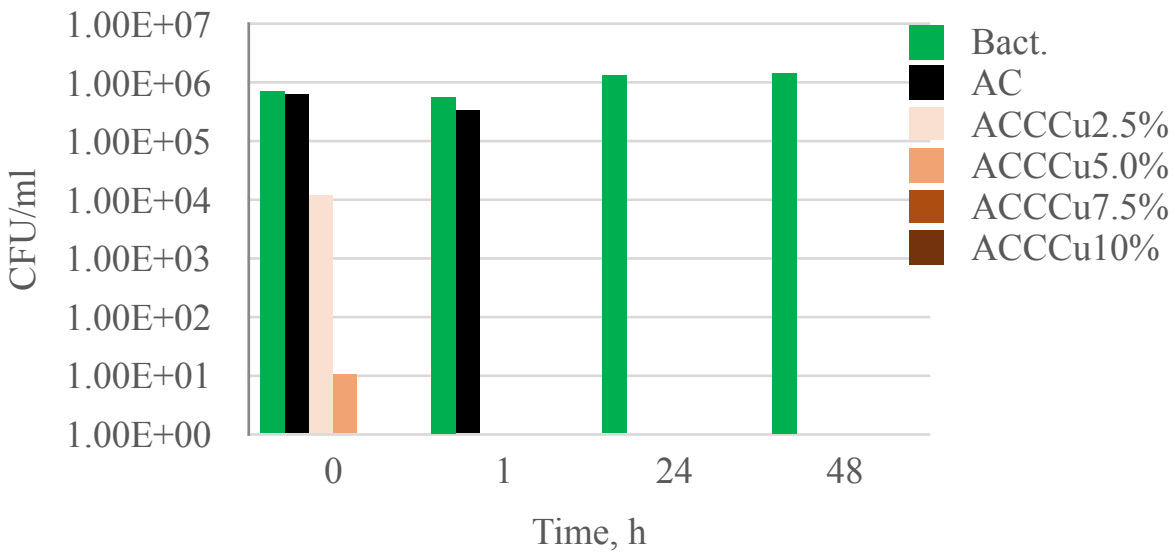


Fig. 30 Antibacterial effect of ACCCu in different concentrations of copper against *E. coli.*

Table 3 provides information on the reduction (% R) of *E. coli* relative to metal concentration at different contact times. The best antibacterial effect against *E. coli* with 100% reduction of the microbial number was observed at the starting point when the concentration of Cu metal in ACC was 7.5% and more. ACC with lower Cu concentration showed the same effect after 1 h.

Table 3 Percentage reduction (% R) of *E. coli* at different concentration of Cu and at different contact time.

Sample	%R (t=0h)	%R (t=1h)	%R (t=24h)	%R (t=48h)
AC	5,7	40	100	100
AC+Cu (2.5%)	98,3	100	100	100
AC+Cu (5,0%)	99,9	100	100	100
AC+Cu (7.5%)	100	100	100	100
AC+Cu (10%)	100	100	100	100

4.5.3. Analysis of the effect of carbon composites obtained by hydropyrolysis on suspensions of *S. aureus* (clinical strain)

The difference in the structure of the cell wall of Gram-positive and Gram-negative bacteria also determines a number of differences in their resistance to antibacterial agents. So far, the impact of metal-carbon composites on a model of Gram-negative bacteria has been examined. To evaluate the impact on Gram-positive bacteria as well, the newly synthesised composites including Ag, Cu and Mg were tested against a clinical strain of *S.aureus*, as this species causes a wide range of infections in all parts of the human body, but can also asymptomatically colonise the skin and nasopharynx (Rigaill et al., 2023).

The patient's throat swab was processed according to good laboratory practice. The isolated *S.aureus* strain was tested for antibiotic susceptibility (Becton Dickinson, BD BBL™) and the antibiogram showed the following results: clindamycin (S), ciprofloxacin (I), erythromycin (S), cefoxitin (S), gentamicin (S), penicillin (S), sulfamethoxazole/trimethoprim (S) and vancomycin (S).

The experiments were carried out at the same exposure times - 0, 1, 24 and 48 hours and the same metal concentrations in the composites - 10%. Again, suspensions containing only the bacterial strain and a bacterial solution with activated carbon were used as controls to ensure that the antimicrobial effect was solely due to the composites.

The results of the experiments performed are depicted in Figure 31, which is a histogram representing the bacterial concentration of *S. aureus* (CFU/ml) as a function of the contact time (0, 1, 24 and 48 hours) with composites synthesised by hydropyrolysis.

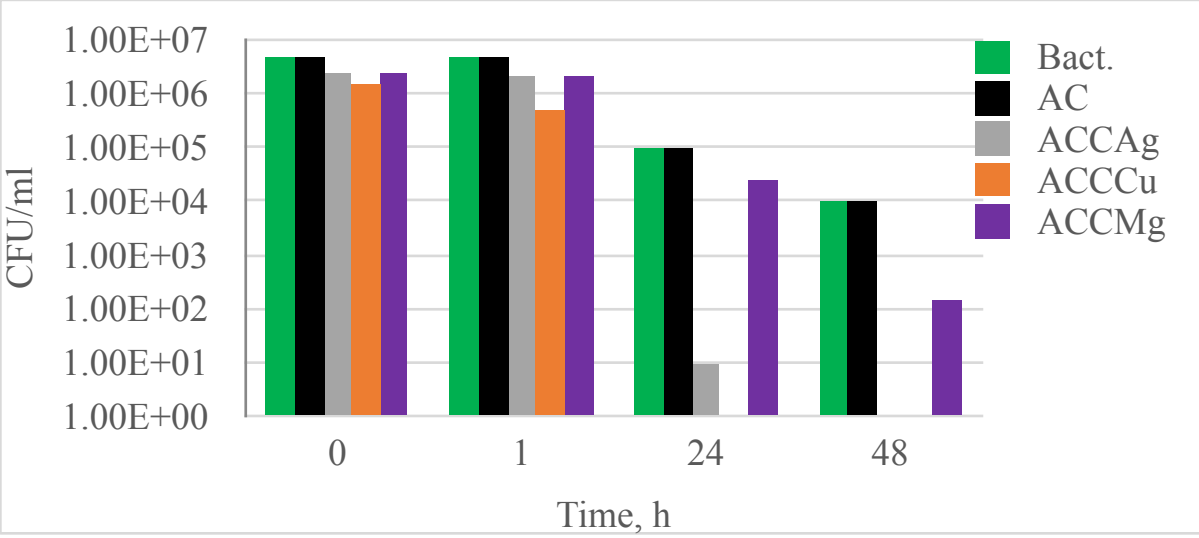
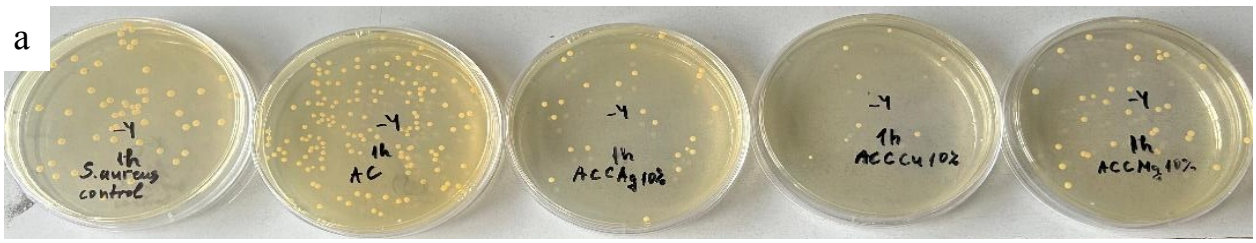


Fig. 31 Antibacterial effect of ACCAg, ACCCu and ACCMg prepared by hydropyrolysis against *S. aureus* clinical strain.

The microbial count of the first and second controls dropped slightly from 10^6 to 10^4 CFU/ml, i.e. at a rate of one log per day, indicating that the bacteria appear to be more vulnerable to the experimental conditions /conditions during the testing period did not provide optimal growth / and there is no interaction between them and the activated carbon. As described above in the previous section, a microbial count method was applied as a measure of the antibacterial effect of the metal composites.

This can be seen in Fig. 32, which is a photograph of selected plated Petri dishes after 24 hours of incubation, when bacterial colonies are visible.

a



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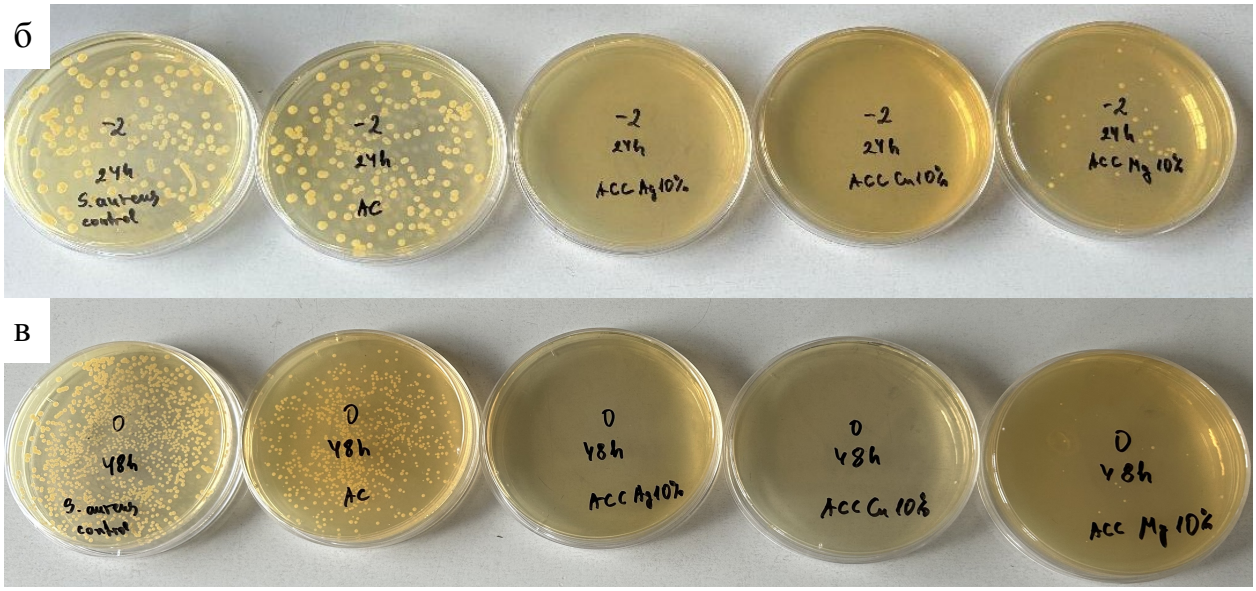

Fig. 32 Microbial count of *S.aureus* in bacterial control, AC control and ACCAg, ACCCu and ACCMg samples at 1 hour and 10000x dilution (a), 24 hours and 100x dilution (b) and 48 hours, without dilution (c).

Each bacterial colony counts as one bacterial cell. The absence of colonies on petri dishes for the ACCAg and ACCCu samples is consistent with their excellent antibacterial potential.

The percent reduction (%R) of *S.aureus* at different contact time was determined relative to the bacterial control at contact time T_x /as for *E. coli/*, and the data are shown in Table 4. Best antibacterial effect against *S.aureus* was observed using 10% ACCAg and ACCCu with a 100% reduction in microbial counts in 24 h, which was slower compared to *E. coli*.

Table 4 Percent reduction (%R) of *S.aureus* at different contact time and at different metal content (Ag, Cu and Mg).

Sample	%R (t=0h)	%R (t=1h)	%R (t=24h)	%R (t=48h)
AC	10	10	0	0
ACCAg	52	56	99.99	100
ACCCu	88	90	100	100
ACCMg	52	56	76	98.6

Another study (Li et al., 2006) that evaluated the minimum inhibitory concentrations of nanoparticles consisting of a mixture of silver nitrate and titanium dioxide against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus*

ATCC 25923 also found that *S. aureus* was more tolerant to nanoparticles. The same was observed in another study (Joshi, 2022.). ACCMg (Tahir, 2023.) also reached 98.6% reduction, but after 48 hours.

The higher antibacterial activity against *E. coli* compared to *S. aureus* may be due to the difference in the structure of the cell wall of these bacterial species, since the former belongs to the group of Gram-negative bacteria, and the latter to the group of Gram-positive bacteria. The latter form a thick peptidoglycan layer, which is an essential barrier for many harmful chemical compounds.

The results show that the antibacterial activity depends on the contact time and the nature of the metal.

Metal nanoparticles are well known for their antibacterial activity and many studies have confirmed their effectiveness as antimicrobial agents when added to a wide variety of materials (Al-Gaashani et al., 2021). However, the mechanism of antimicrobial activity is not fully understood. Silver has been known since ancient times for its antimicrobial properties, and the antibacterial effect of silver materials has received widespread attention due to the low toxicity of silver cations to human cells (Williams, 1987). Using transmission electron microscopy, it was demonstrated that silver nanoparticles can attach to and penetrate *E. coli* cells and can also damage the cell membrane (Choi et al., 2008). On the other hand, Ag^+ ions released from silver-based materials have been reported to interact with *E. coli* respiratory chain enzymes and inhibit the respiratory chain, leading to cell death (Rai et al., 2009). Marambio-Jones and Hoek, 2010 suggested that the most common bactericidal mechanisms of silver-based materials are: (i) uptake of free silver ions followed by disruption of ATP production and DNA replication, (ii) silver particles and silver ions in the presence of dissolved oxygen generate reactive oxygen species that attack membrane lipids and lead to disruption of membrane and mitochondrial function or cause DNA damage causing bacterial cell death, and (iii) silver particles directly damage cell membranes. Production of reactive oxygen species (ROS) (Muñiz Diaz et al., 2021) and changes in cell membranes (Stoimenov et al., 2002) are the likely bactericidal mechanisms of Mg nanoparticles. There are many studies that reveal a strong bactericidal effect of Cu nanoparticles (Shaffiey et al., 2014; Motlatle et al., 2016; Babaei et al., 2018). Copper nanoparticles release ions and ROS that lead to lipid peroxidation and cause damage to phosphorus- and sulfur-containing biomolecules, leading to disruption of intracellular biochemical processes (Saraeva et al., 2022). Some studies have shown that the bactericidal properties of nanoparticles are size-dependent, as the only nanoparticles that present a direct interaction with bacteria preferably have a diameter of ~1–10 nm (Morones et al., 2005). Our results show that the investigated materials contain Ag, Cu and Mg nanoparticles in the same range ~ 1–10 nm and the presence of Ag^0, copper in Cu^+ and Cu^{2+} coordinations in a ratio of 2:1, Mg^{2+}. Therefore, we expect that they provide the same mechanism as discussed above.

Conclusions

1. In the conducted experiments with aerobic reference bacterial cultures, the best antimicrobial effect of the tested photosensitizers was observed using gallium phthalocyanine.

2. On the tested anaerobic reference bacterial cultures, the strongest effect of the action of the photosensitizers was found using methylpyridyloxy zinc phthalocyanine and p-tetra-mercaptopyridine zinc phthalocyanine.

3. Zinc phthalocyanine has been shown to be most effective in the treatment of reference yeast cultures.

4. Clinical aerobic bacterial isolates tested were affected in the highest rate with zinc phthalocyanine application, but the impact compared to the reference strains was weaker.

5. The impact on clinical anaerobic bacterial isolates was most notable with tetra-methylpyridyloxy zinc phthalocyanine, p-tetra-mercaptopyridine zinc phthalocyanine and octa-mercaptopyridine zinc phthalocyanine, the effect being compared with that of reference strains was stronger.

6. In experiments with biofilms, the best effect was observed when using silicon phthalocyanine on a 48-h *E. faecals* biofilm .

7. The newly synthesised composite materials have a very good potential for application as antibacterial agents, especially the copper composite.

8. The method of obtaining the activated carbon does not significantly affect the antibacterial properties. When applying the different methods we found close values for their impact, which is most noticeable in the case of one-stage hydropyrolysis.

9. Best result or 100% reduction of the microbial count of *E. coli* in the first minutes was observed when composites were used at a copper concentration of 7.5% and higher.

10. The obtained results show that the newly synthesised composite materials have very good potential for application as antibacterial agents against staphylococci (*S. aureus*), especially the copper composite.

11. The physico-chemical characterization of the composites confirmed the metal content and showed a carbon matrix with very good absorption properties and low content of impurities.

12. The composites of activated carbon with metal nanoparticles combine properties of metals and activated carbon, which contributes to increase the quality of the final product and the application of its antibacterial properties, for example in hygiene devices and individual masks.

Contributions

1. The application of photodynamic inactivation with metal-containing phthalocyanine photosensitizers is a new optimized alternative to the methods used today in the fight against bacterial infections in the oral cavity and their prevention.

2. Carbon composites were synthesised using a new ecological technology from waste products as precursors.

3. The combination in the metal-containing carbon composites of properties of metals and activated carbon contribute to the increase the qualities of the desired product and expanding its antibacterial properties applications .

4. The results obtained in the *in vitro* experiments can be used for the preparation of a protocol for further *in vivo* experiments.

5. The use of photosensitizers and newly synthesised activated carbon composites is a new approach for reducing excessive use of antibiotics in unison with the modern trend for overcoming antibiotic resistance.

6. The development of new antibacterial materials is an important step in the fight against pathogenic organisms in humans and in the environment.

List of publications related to the dissertation:

1. Angelov I, V Mantareva, V Kussovski, D Worle, H Kisov, M **Belcheva**, T Georgieva, S Dimitrov. Susceptibility of representative dental pathogens to inactivation by the PDT with water-soluble photosensitizers. LAT 2010: International Conference on Lasers, Applications, and Technologies. Edited by Panchenko, Vladislav; Mourou, Gérard; Zheltikov, Aleksei M. Proceedings of the SPIE. 2011;7994 1A:7994-45. IF 0.440, SJR 0.24

2. **Belcheva** M, Bonchev A, Tsenova I, Vasileva R. Effect of photodynamic therapy on Staphylococcus aureus, Enterecoccus faecalis and Candida albicans - causes of infections in the maxillofacial region. Overview. Problems of dental medicine. 2012;1(38):42-49.

3. **Belcheva** M, G Georgiev, B Tsyntsarski, U Szeluga, L Kabaivanova. Antibacterial properties of metal nanoparticles – incorporated activated carbon composites using waste biomass as a precursor. Journal Diamonds and Related Materials. 2024;141:110545. doi.org/10.1016/j.diamond.2023.110545. Q2 (2023) IF 4.3 (2023), SJR(2023) 0.67

Total impact factor: 4.74

Noticed citations of the publications related to the dissertation:

1. Angelov I, V Mantareva, V Kussovski, D Worle, H Kisov, M **Belcheva**, T Georgieva, S Dimitrov. Susceptibility of representative dental pathogens to inactivation by the PDT with water-soluble photosensitizers. LAT 2010: International Conference on Lasers, Applications, and Technologies. Edited by Panchenko, Vladislav; Mourou, Gérard; Zheltikov, Aleksei M. Proceedings of the SPIE. 2011;7994 1A:7994-45.

cited in:

1. Elganzory H, M Arief, M Amine, El-Z Ebeid. Microwave-assisted Solvent-free Synthesis and Fluorescence Spectral Characteristics of some Monomethine Cyanine Dyes. Journal of Chemical and Pharmaceutical Research. 2014; 6:143-161.

2. **Belcheva** M, G Georgiev, B Tsyntsarski, U Szeluga, L Kabaivanova. Antibacterial properties of metal nanoparticles – incorporated activated carbon composites using waste biomass as a precursor. Journal Diamonds and Related Materials. 2024;141:110545. doi.org/10.1016/j.diamond.2023.110545. Q2 (2023) IF 4.3 (2023), SJR(2023) 0.67

cited in:

1. Ramli MR, NF Shoparwe, MA Ahmad, MF Yusop. Acetaminophen removal using porous activated carbon derived from corn cob: optimization and mass transfer modelling. Journal of Chemical Technology & Biotechnology. 2024; 99(9):2088-106.

Total number of citations: 2. (SCOPUS)

Participation in grant projects and scientific events related to the dissertation

Scientific events:

1. Angelov I., V. Mantareva, V. Kussovski, D. Worle, H.Kisov, M. **Belcheva**, Tz. Georgieva, S. Dimitrov. Susceptibility of representative dental pathogens to inactivation by the PDT with water-soluble photosensitizers LAT 2010: International Conference on Lasers, Applications, and Technologies. (2010).

2. **Belcheva** M., I. Angelov, V. Mantareva, V. Kusovski, L. Kabaivanova, B. Tsintsarski. Photodynamic inactivation of oral pathogens. Third Interdisciplinary Doctoral Forum 2022, June 6-7, Kyustendil, Bulgaria.

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