SYNTHESIS AND TOXICITY STUDY ON AQUATIC ORGANISMS OF PAINT WITH TITANIUM DIOXIDE MODIFIED WITH Ca AND Cu IONS

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The aggravation of the phenomenon of antibiotic resistance of pathogenic microorganisms is forcing the industry to discover new biocidal compounds with the role of combating healthcare-associated infections (HAIs) [1, 2, 3, 4] . The pigment based on TiO_2 decorated with Ca and Cu ions, which is presented preparation here, was evaluated by performing the following tests : degradation test (determination of chemical oxygen consumption CCo-Cr), test of inhibiton of the growth of freshwater algae (using unicellular green algae, sp. Selanastrum capricornutum), te acute toxicity test with planktonic crustaceans (using water fleas,Daphia) and the acute lethal toxicity bioassay with aquatic microorganisms (carp fry). The dates were obtained that attests that the pigment tested is harmful for long-term aquatic life, being non-biodegradable.

Keywords: TiO_2, degradation, inhibition, toxicity, lethal

1. Introduction

Patient care areas, all tangible surfaces in the hospital, but also indoor air, are the most important reservoirs of pathogens, with direct implications in the way infections are transmitted from one person to another [5]. Active disinfection and cleaning methods have often been found to be ineffective in mechanically and chemically removing pathogens. The cause would be the development of bacterial biofilms on surfaces that have a major role in the dissemination of pathogens and the occurrence of healthcare-associated infections (HAI) [1, 2, 3, 4].

It has been found that one of the best measures to prevent infections caused by infection is to block the development of biofilms on medical surfaces. Simultaneously with the development of new active disinfection methods (discovery of new disinfectants, use of automatic disinfection and ozonation equipment, implementation of UV-C robots), special emphasis was placed on the discovery and use of antimicrobial coatings for medical surfaces exposed to high

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risk of contamination [6].

Passive protection measures are implemented in the form of antimicrobial and anti-adhesive coatings on various critical surfaces [7]. Very good results have been obtained by applying these antimicrobial and anti-adhesive coatings to the surfaces of medical materials or to constructed surfaces that may be contaminated with pathogens [8]. There are four main strategies for obtaining antimicrobial surfaces: (i) release of antimicrobial agents (depending on the leaching of incorporated antimicrobial agents); (ii) mechanical damage to the membrane of pathogens by contact (cell membranes are disrupted by contact with immobilized compounds); (iii) use of low-energy surfaces to inhibit microbial adhesion and biofilm development on the surface (immobilization of molecules such as polyethylene glycol and zwitterion that can inhibit cell wall protein adsorption);

(iv) the use of light-activated coatings (generation of reactive oxygen species (ROS) such as singlet oxygen and hydroxyl radicals by photosensitizers that can degrade microbial pathogens, causing damage to their DNA or cell membrane) [9].

Persistent antimicrobial properties have the potential to reduce the microbial load on clinical surfaces [10,11]. However, these coatings have not spread to the medical environment due to unknown emissions and ecotoxicological effects [12]. There are no risk-benefit analyses, no unitary regulatory system approved by regulatory authorities, and the lack of clear evidence of benefits and risks may lead to the loss of the potential of these coverage models [10, 13,14,15,16].

Currently, a number of biocidal compositions are used with real success, in the form of washable paints, protective resins and fabrics and linen, in which antimicrobial agents of the type 1,2-Benzothiazol-3(2H)-one (CAS no. . 2634-33-5), triclosan (CAS no. 3380-34-5), various polymers, copper or silver [17]. Although these measures have proven their usefulness, they present several shortcomings caused by the active bio-toxic agents contained in the matrix of these compositions.

The main problem is the sublimation of active bio-toxic agents in the matrix of the composition, because they are either active eluting agents (ex, ionsor nanoparticles of silver, copper, zinc, or antibiotics, chloride, iodine, etc.), or immobilized molecules that become active at contact (eg quaternary ammonium polymers or peptides) [18]. As a result, the biocidal effect of these compositions decreases with application and soon becomes ineffective in controlling pathogens. The second major problem is related to the high toxicity of active biotoxic agents. Sublimation produces toxic fumes that can be inhaled. Numerous cases ofskin allergies caused by prolonged exposure to 1,2-benzothiazol-3(2H)-one (CASno. 2634-33-5) have been reported [17,19]. There is a call for European control authorities to limit the use of 1,2-benzothiazol-3(2H)-one (CAS no. 2634-33-5) in various products to eliminate the risk of skin allergies in antimicrobial formulations. Silver has been used since ancient times as an antibacterial product. New types of

silver-polymer composites are based on the incorporation of silver nanoparticles into various substrates. Microbial resistance to silver is known to exist, particularly Gram-negative bacteria, and not Gram-positive bacteria. This resistance to silver can be genetically encoded in chromosomes or in transferable plasmids to other bacteria [20].

Achieving antibacterial protection of surfaces at risk of pathogen contamination required the production of photocatalytic antimicrobial paint that can be used as an environmentally friendly coating solution with high long-term efficiency. This is a versatile solution that ensures the decoration of different surfaces but also antibacterial protection.

In previous studies [21], we presented a method for obtaining and characterizing a layer of composites based on microparticles of TiO_2 with antimicrobial properties, having a great potential in limiting the growth and development of pathogens. The materials obtained can be applied in the form of a thin film on masonry, plastic or metal surfaces and present an effective antimicrobial effect after at least 2 hours of exposure to various microorganisms.

This study presents the following: a method for obtaining a TiO_2 based composite decorated with calcium and cooper ions and the evaluation of the ecotoxicity of the obtained composite by performing toxicity tests on freshwater algae – *Selenastrum capricornutum*, on planktonic crustaceans- *freshwater fleas, Daphnia magna species* and aquatic organism – *Carp fry*, respecting the standards.

2. Experimental

2.1. Synthesis of the TiO_2 composite decorated with Ca and Cu

The method of obtaining the new composite is based on patent RO136026 (W2022/220702 A2) and has been described in detail in previous studies.

We used only raw materials of industrial origin, certified according to ISO 18451-1: 2019-pigments, dyes and diluents.

For the manufacture of sample were used: TiO_2 rutile (CAS 13463-67-7) produced by Tronox, the industrial product NaOH flakes (CAS 1310-73-2) was purchased from CIECH Soda Romania and from BRENNTAG Romania $CaCl_2$ (CAS 10043-52-4) and $CuSO_4 \cdot 5H_2O$ (CAS 7758-98-7 pentahydrate).

TiO_2, Tytanopol microparticles and $Ca(OH)_2$, $CuSO_4$ and NaOH were used as raw material for pigment preparation. The process takes places in two stages

A) Decoration stage with calcium oxides

In a Berzelius glass, dissolve 4g of NaOH in 100 ml of distilled water. Then 10g of rutile TiO_2 are added to the solution – and this is called solution A. In this solution, 5.5 g of $CaCl_2$ are added and heated. The reactions that take place in this stage can be described as follows:

$$CaCl_2 + 2NaOH = Ca(OH)_2 + 2NaCl$$

$$Ca(OH)_2 = CaO + H_2O \text{ (at temperature)}$$

B) In stage two, the decoration with cooper oxides is carried out. For this stage, two solutions are prepared separately :

- 15 ml of 0.5M NaOH solution – called solution B
- Dissolve 0.78g of $CuSO_4 \cdot 5H_2O$ in 25ml of distilled water – called solution C.

Solutions B and C are stirred separately and then solution B is added over solution C. A blue-green precipitate of $Cu(OH)_2$ is obtained. The $Cu(OH)_2$ solution is then poured over solution A with stirring and heat to boiling. After boiling, let the solution settle for 24 hours. The excess water is removed and a suspension of TiO_2 decorated with metallic oxides of cooper and calcium is obtained.

2.2. Materials and Methods

To evaluate the ecotoxicity of the product, the following tests were performed Toxicity test to determine the adverse effects of a substance on the growth of the freshwater microalga Selenastrum capricornutum using the experimental procedure from the OECD guide 201 and the national standard SR EN ISO 8692:2012.Acute toxicity test with planktonic crustaceans (water fleas, Daphnia magna species) that complies with the experimental procedure of the OECD 202 guide and the SR EN ISO 6341: 2013 standard. Lethal toxicity test with aquatic organism (carp fry) according to the OECD 203 guide and the SR EN ISO 7346-1: 2004/C91:2008 standard.

3. Results and Discussion

3.1. Ecotoxicity test

Visualization of algal growth under the action of the tested product in the range of nominal concentrations of 0 – 100 mg product / liter, in 3 replicates (C_0-0-mg/L; C_1-100mg/L; C_2-50mg/L; C_3-25mg/L; C_4-12.5mg/L; C_5-6.25mg/L).

Biomass growth was measured as optical density at a wavelength of 670 nm for each concentration of tested product and control, for the entire experimental period. Algal toxicity experiments revealed the following: The pigment sample causes a significant inhibition of the algal growth rate compared to the control at concentrations of 50 and 100mg/L. In the range 6.25 – 25 mg/L, an inhibition $\leq 25\%$ is observed, considered insignificant. The specific growth rates at 72h remain within the limit of the control up to a concentration of 25mg/L and decrease significantly compared to the control at the concentrations of 50mg/L, respectively 100mg/L. The visual analysis of algal growth in the test tanks confirms the results presented above by the variation in the color of the chlorophyll pigment, which represents the growth of algal biomass directly proportional to the test concentration. The toxic

effects of inhibiting algal growth are evident at concentrations ≥ 30 mg product/L.

The acute toxic value of the tested pigment was estimated at 36.06 mg/L (95% confidence interval 31.87 – 41.38 mg/L).

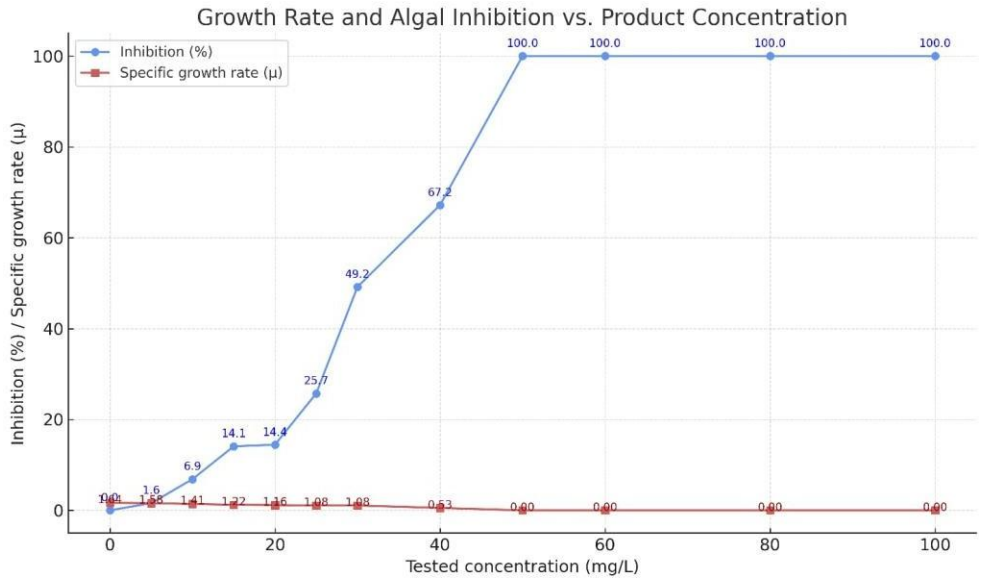

Fig. 1. Graphic representation of specific growth rates (μ) and algal growth inhibition (%) for each tested product concentration at 72 h.

The acute toxicity test on algae carried out in the laboratory met all the criteria specified in the standardized work methodology, respectively:

The control samples (the algal solution without the test product) showed a progressive increase in the optical density, i.e. the number of algal cells, depending on the incubation time, so that at the end of the experiment (72 hours) the average value of the rate of daily growth (μ) was 1.64.

The pH value in the control solutions did not show variations >1 pH unit.

The coefficient of variation of the growth rate of the algae cultures from the three replicates of the blank control sample did not exceed the 5% value imposed as a limit in the test method.

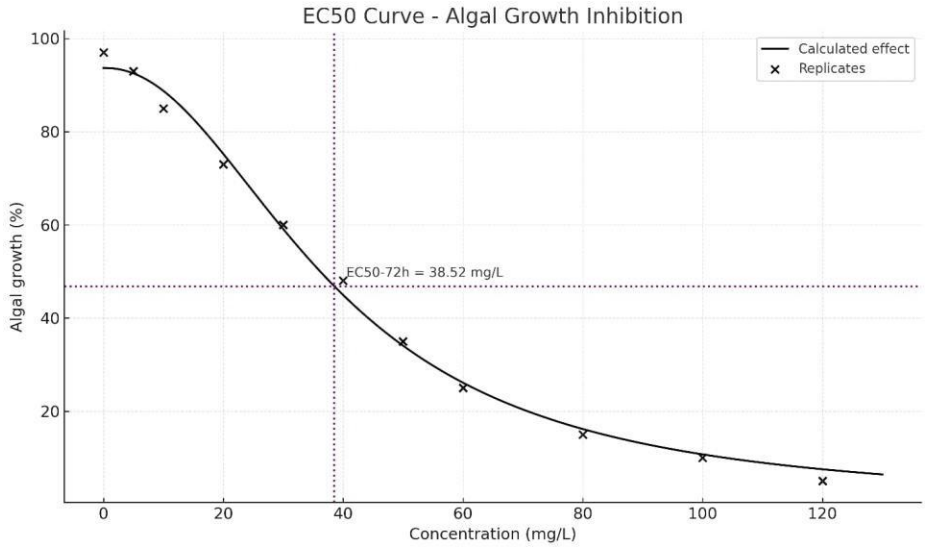

Fig. 2. EC_{50} estimation graph for the pigment teste at 72 hours

3.2. Acute toxicity test with planktonic crustaceans

During the 48h acute toxicity experiments with daphnia, the monitoring of the oxygen regime, temperature and pH in the test system was carried out:

Test: initial pH 9.81-8.13 pH units, final 7.78- 8.8 pH units, dissolved oxygen initial 8.10 – 8.25 mg O2/l, final 7.70 – 8.20 mg O2/l, initial temperature 23.5 – 24 °C, final 23.3-23.8 °C. Control: initial pH 7.90 – 8.04 pH units, final 7.58-7.60 pH units, dissolved oxygen initial 7.78– 7.83 mg O2/l, final 7.25– 7.42 mg O2/l, initial temperature 20.6 – 21 °C, final 20.6-20.8 °C.

The pigment tested causes toxic effects of inhibition / mortality between 15 – 100% in the concentration range 17.5 -100 mg/L, the highest effect being recorded (95% and 100%, respectively). At concentrations lower than 17.5 mg/L, the effects are considerably reduced to zero. Mortality effects are due to ingestion of the test compound that affects the organisms' physiological systems, but also to compound deposits on the fore and hind antennae that prevent the organism from moving in the water mass. The tested product concentrations were graphically represented according to the recorded immobilization/mortality effects and the value of the concentration that inhibits the number of tested organisms ($EC_{50\text{-}48h}$) was estimated.

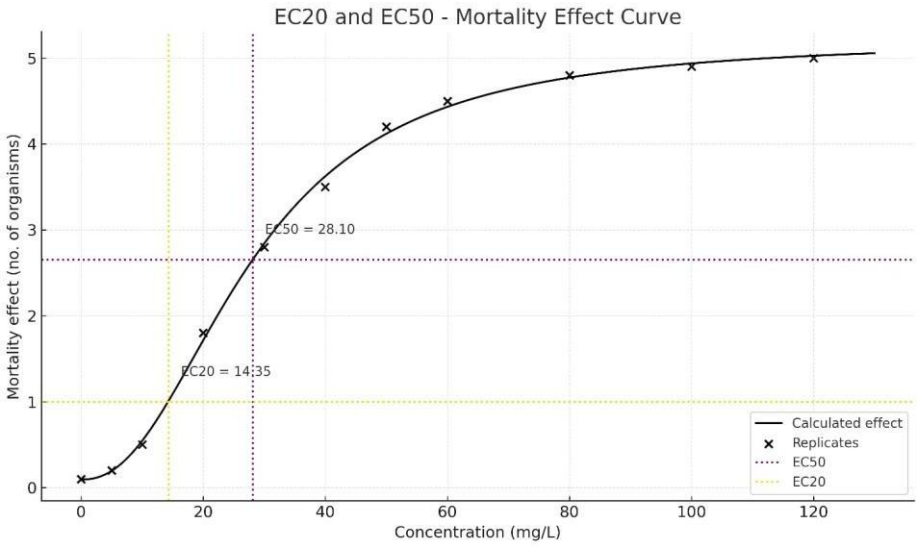

Fig. 3. $EC_{50\text{-}48h}$ estimation graph for the tested pigment

From the point of view of validity, the acute toxicity test carried out in the laboratory fulfilled all the criteria specified in the standardized work methodology for the blank test: the dissolved oxygen concentration at the end of the test was >4 mg/L, respectively 7.25 mgO_2/L the percentage of immobilization was 0%.

3.3. Lethal toxicity test on freshwater fish (Carassius auratus gibelio species)

The objective of the study is to establish the value of the average lethal concentration of the product / chemical substance (LC_{50}) that causes mortality for 50% of the group of aquatic organisms tested - freshwater fish (carp fry). The main objective of the tests was to observe if sublethal effects occur or if mortality is recorded for the aquatic organisms (fish) used in the acute toxicity experiments performed with the tested pigment.

Table 1.

Toxic effects recorded in experimental tests with juvenile fish

Tested product (mg product/liter)	Number of fish used in the test	Mortality at 96h %
Replicated 1-100 mg product/ liter (0.5012g product dissolved directly in 5l dilution water)	5	0
Replicated 2-100 mg product/ liter (0.5002g product dissolved directly in 5l dilution water)	5	0

BLANK Dilution water without test product	5	0
Product toxicity	LC50 (96h) = >100 mg/ liter **NON-TOXIC product**	

There were no mortalities at the tested concentration (100mg/l). The test solutions showed an opalescent color due to the test product in suspension. During the visual inspection of the fish subjected to testing, they showed hypoactivity.
Acute toxicity testing with juvenile fish revealed:

- The concentration of 100mg product/L did not cause mortalities of the biological material used - freshwater fish, species Carassius auratus gibelio, which classifies the tested product, the pigment, as NONTOXIC - $_{CL50(96h)}$ =>100mg/l.
- Evaluation of the results obtained in the degradability assessment test (based on the C_{BO5}/CC_{OCr} ratio) and acute toxicity tests with aquatic organisms. Classification and Labeling of Chemicals, 2011 Revision; REACH 1907/2006 amended / supplemented whereby Regulation (EC) 1272/2008 and Regulation (EU) 286/2011, classification categories and hazard phrases of chemical substances / preparations based on acute lethal concentrations / inhibitory concentrations, experimentally determined for the body aquatic(fish/daphnia/algae), are:
  - Acute toxicity category 1
  - Very toxic - acute (short-term) hazard for the aquatic environment:
    - $LC_{50\text{-}96h}$ (for fish) / $CE_{50\text{-}48h}$ (for crustaceans) / $CEr_{50\text{-}72h}$ or $_{96h}$ (for algae or other aquatic plants) ≤ 1 mg/l (risk phrase: H 400- very toxic to aquatic life).
  - **Chronic toxicity category 1** (reference to the algae toxicity test which can be considered a chronic test).
  - Long-term danger for the aquatic environment: $EC_{50\text{-}48h}/CrE_{50\text{-}72h}$≤ 1mg/l and the substance is not rapidly degradable (risk phrase: H410 – Very toxic to aquatic life with long lasting effects).
- **Chronic toxicity category 2:**
  - long-term danger for the aquatic environment: 1mg/l<$CL_{50\text{-}96h}/CE_{50\text{-}48h}/CrE_{50\text{-}72h}$≤10mg/l and the substance is not rapidly degradable (risk phase: H411- TOXIC for aquatic life, with long-lasting effects).
- **Chronic toxicity category 3**
  - long-term danger for the aquatic environment: 10mg/l<$CL_{50\text{-}96h}/EC_{50\text{-}48h}/CrE_{50\text{-}72h}$≤100 mg/l and the substance is not rapidly degradable(risk phase: H 412 – HARMFUL to aquatic life, with

long-lasting effects) NON-TOXIC for the aquatic environment $CL_{50}/CE_{50-48h}/CrE_{50-72h}$> 100mg/l and the substance is biodegradable.

Taking into account the classification categories highlighted above and the toxicity values obtained in the range 10mg/l< EC_{50-48h}/ CrE_{50-72h}≤ 100 mg/l for crustacean invertebrates (Daphnia magna species) and green algae (Pseudokirchneriella species subcapitata), as well as the fact that the tested pigment has no degradation capacity (CBO5/CCO-Cr ratio < 0.1) it can be appreciated that it presents a long-term danger for the aquatic environment, Category 3 of chronic toxicity (H 412) - harmful to aquatic life, with long-lasting effects.

4. Conclusions

The two composite models based on TiO_2– the pigment contains copper ions and calcium chemically bound to the surface of an ionic structure of TiO_2. The antibacterial effect of the TiO_2-based composite is due to the intrinsic chemical properties of this pigment and not the release of heavy metal ions

The tested pigment has an acute toxic value ECr_{50-72h} = 36.06 mg/L (95% confidence interval 31.87 – 41.38 mg/L) for green algae sp. *Selenastrum capricornutum*, which reveals a potential harmful effect on the test organisms.

The estimated value of the average lethal concentration for planktonic crustaceans, the species Daphnia magna was EC_{50-48h} = 36.10 mg product / liter (confidence interval 31.08 – 42.29 mg/L).

Regarding the effects on vertebrate organisms, namely juvenile fish species *Carassius auratus*, *gibelio*, the tested pigment is classified as non-toxic for the aquatic environment LC_{50-96h} >100 mg/l and the product is not biodegradable. There were no mortalities at the tested concentration (100mg/l). Solutions to test have presented opalescent color due to the tested product in suspension. On visual inspection of the tested fish, they showed hypoactivity.

Following the tests carried out and the results obtained, it was decided that the pigment obtained and tested is harmful to green algae, it is toxic to planktonic crustaceans, the species presented above, but due to the fact that it attaches to the antennae of the analyzed species resulting in death. In the case of vertebrates, as can be seen from the results presented, the pigment is classified as non-toxic, but presents a danger because it is not biodegradable.

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