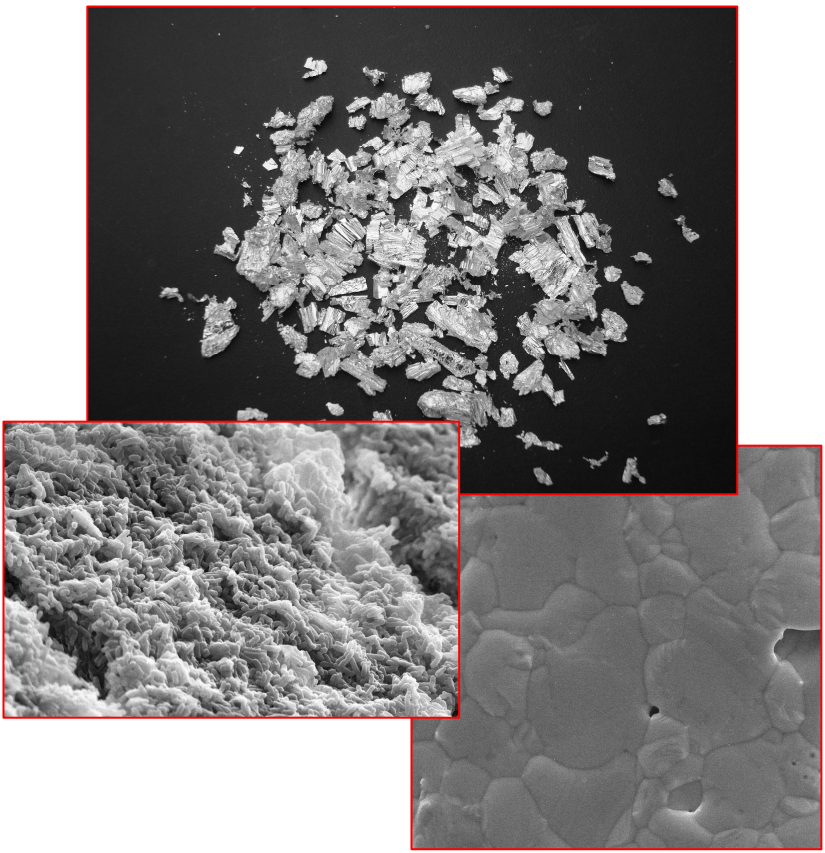
RESEARCH AND DEVELOPMENT OF NEW PRODUCTS AND PROCESSES: RECLAMATION OF A MANUFACTURING WASTE



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RESEARCH AND DEVELOPMENT OF NEW PRODUCTS AND PROCESSES: RECLAMATION OF A MANUFACTURING WASTE

PhD Thesis

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Chapter 1

Introduction

The aim of the research was the development of new products and processes from a manufacturing waste.
The manufacturing waste were produced by a metallurgic Italian company which produced thin silver metallic films. These films were used as coating and food decoration.
Thin films were produced by an hammering process. The starting material, silver laminate, had a thickness of 10 μm. During the hammering process films with various thickness were obtained. The final produced silver films had a thickness of about 300 nm which can be considered a submicrometric dimension, near to the nanometric range (≤100 nm). At the end the submicrometric films were cut, giving a square shape (10 cm x 10 cm) and inserted into boxes. The residues, produced during this step, named untreated flakes (UF) (figure 1), were used in the research.

Figure 1 – *Manufacturing waste: untreated silver flakes*.

Mainly we studied the possible use of UF as a catalyst in antimicrobial applications in aqueous medium.

1.1 Structure of the research

After a thorough bibliographic research considering the various aspects of the use of silver as antimicrobial agent (chapter 2), the research was divided in various stages:

- To investigate the use of flakes as antimicrobial agent, at first we verified if they possessed antimicrobial capability. We conducted some antimicrobial tests using *Escherichia coli* (*E. coli* JM109) as Gram-negative microorganism model. We showed that the flakes (UF) didn't present antimicrobial capability up to 24 h (chapter 3).

- On the basis of these results we treated the UF with three activation processes:
  - Thermal activation in reducing atmosphere;
  - Thermal activation in air;
  - Chemical activation with H_2O_2.

  Then we tested the antimicrobial activity of the new obtained products. We showed that the activation processes gave the flakes antimicrobial capability (chapter 3).

- The antimicrobial agents are classified as bacteriostatic or bactericidal. Bacteriostatic agents cause only the growth inhibition of microorganisms while bactericidal agents cause the cells death. *E. coli* were observed at a fluorescence microscope and at a scanning electron microscope (SEM), after specific treatment processes. In this way we determined that activated flakes were bactericidal agents (chapter 4).

In the next two stages we investigated the possible mechanism of action of the flakes:

- On the basis of literature knowledge we studied the relationship between the capability of flakes to release silver in aqueous medium and their antimicrobial activity. We showed that the presence of flakes was necessary to have *E. coli* decrease over time (chapter 5);
- We characterised the morphology and chemical composition of the flakes surface by SEM and XPS to verify if the antimicrobial activity acquired by activated flakes could be affected by some morphological or chemical change. We concluded that the antimicrobial property was due to the presence of dissolved oxygen (chapter 6).

- In the final stage we performed some experiments which simulated possible applications of activated silver flakes (chapter 7).

Chapter 2

Use of Silver as Antimicrobial Agent: State of the Art

2.1 Metallic silver

The antimicrobial effects of silver (Ag) have been known for thousand of years. In ancient times, it was used in water containers and to prevent putrefaction of liquids and foods. In ancient time in Mexico, water and milk were kept in silver containers. Silver was also mentioned in the Roman pharmacopoeia of 69 B. C. [1].
At the beginning of last century, Gibbard (1937) was the first to systematically investigate the antimicrobial properties of silver [2].

2.2 Silver ions

Silver nitrate is the most common silver compound used as an effective medicine in clinical practice. There is strong evidence in the literature that the active component of this silver salt is the silver itself [3]. Silver nitrate is a substance that releases silver ions rapidly.
The antimicrobial activity of silver ions dissolved as silver nitrate was studied in different works. Hwang et al. [4] estimated the bactericidal ability at various concentrations (0-0.1 mg/L of Ag^{+}) against different bacterial strains (*Legionella pneumophila*, *Pseudomonas aeuriginosa*, *Escherichia coli*) demonstrating that silver showed sufficient bactericidal ability to inactivate these bacteria at concentrations that did not affect the quality of drinking water or mammalian cells. Zhao and Stevens [5] presented an evaluative model, studying different parameters (CIC, PAE, CAE, MBC …), as a reference for the quantitative analysis of the susceptibility of bacteria to silver ion. Spacciapoli et al. [6] investigated the use of silver nitrate for the treatment of periodontal pathogens. They found silver nitrate more efficient than antibiotics for the treatment of oral cavity of periodontal infections.
Furthermore some Authors investigated the use of Ag^{+} generated electrically. Berger et al. [7] determined the bacteriostatic and

bactericidal concentrations (MIC, MBC) of electrically generated silver against 16 clinical isolates and standard test organisms and they demonstrated that Ag^+ generated at the anode seemed to be a very effective bactericidal agent without any detrimental effects upon normal mammalian cells. Jung et al. [8] investigated the antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* bacteria of a silver ion solution generated from a laundry machine. The *S. aureus* bacterial count was significantly reduced by the silver laundry machine with detergent in comparison to the results with the conventional laundry machine. All of the inoculated *E. coli* bacteria were eliminated when detergent was used in both the silver and conventional laundry machines. In the absence of detergent, *E. coli* was significantly reduced by the silver laundry machine in comparison to the results with the conventional laundry machine.

2.3 Silver nanoparticles

The application of nanoscale materials and structures, usually ranging from 1 to 100 nanometers (nm), is an emerging area of nanoscience and nanotechnology.

Chemical reduction is the most frequently applied method for the preparation of silver nanoparticles (Ag NPs) as stable, colloidal dispersions in water or organic solvents [9, 10]. Commonly used reductants are borohydride, citrate, ascorbate, and elemental hydrogen [12-19]. The reduction of silver ions (Ag^+) in aqueous solution generally yields colloidal silver with particle diameters of several nanometers [10]. Initially, the reduction of various complexes with Ag^+ ions leads to the formation of silver atoms (Ag^0), which is followed by agglomeration into oligomeric clusters. These clusters eventually lead to the formation of colloidal Ag particles [19].

When silver is prepared in the form of very small particles, it is expected to show better antimicrobial characteristics because of their larger specific surface area [20].
With the development of nanotechnology, different works about the bactericidal activity of silver nanoparticles were reported in literature. The unique properties of Ag NPs have been extended into a broader range of applications. Incorporation of Ag NPs with other materials is an attractive method of increasing compatibility for specific applications.

2.4 Mechanisms of action

The exact mechanism of action of silver on the microbes is still not known but the possible mechanisms of action of metallic silver, silver ions and silver nanoparticles have been suggested according to the morphological and structural changes found in the bacterial cells.

Silver ions

Different works reported the mode of action of silver ions in $AgNO_3$ solution.
The most widely known bactericidal mechanism of the silver ion is its interaction with the thiol groups of the L-cysteine residue of proteins and consequent inactivation of their enzymatic functions [21, 22]. Feng et al. [23] studied the effect of silver nitrate against two strains of bacteria (*S. aureus* and *E. coli*) in LB medium by transmission electron microscopy (TEM) and X-ray micro-analysis. They suggested a possible mechanism of action of silver ions. The silver ions enter into the bacterial cells by penetrating through the cell wall and consequently turn the DNA into condensed form which reacts with the thiol group proteins, resulting in cell death. The silver ions also interfere with the replication process. Holt and Bard [24]

using an electrochemical method studied the antimicrobial effect of micromolar concentration of $AgNO_3$ ($\leq 10\mu M$) against *E. coli*. They observed that the rate of respiration increased initially upon the addition of silver(I) because of the uncoupling of the respiratory chain, followed by cessation of respiration.
Low concentrations of Ag^+ induced a massive proton leakage through the *Vibrio cholerae* membrane, which resulted in complete deenergization and cell death [25]. Yamanaka et al. [26] investigated the antibacterial efficacy of silver ions using *E. coli* as a model organism with the help of energy-filtering TEM (EFTEM), two dimensional electrophoresis (2-DE) and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). They concluded that bactericidal action of silver ions was basically due to the interaction of silver ions with ribosome and the suppression of enzymes and proteins necessary for ATP production. Park et al. [27] studied bactericidal activity against *E. coli* and *S. aureus*. They demonstrated that antimicrobial activity of silver ions is closely related to the presence of oxygen which causes the generation of reactive oxygen species (ROS).

Silver nanoparticles

Sondi and Salopek-Sondi [28] reported antimicrobial activity of silver nanoparticles against *E. coli* as a model for Gram-negative bacteria. From the SEM micrographs, they observed formation of aggregates composed of silver nanoparticles and bacterial cells death. They also observed that the silver nanoparticles interacted with the building elements of the bacterial membrane and caused damage to the cell. The TEM analysis and EDAX study confirmed the incorporation of silver nanoparticles into the membrane, which was recognized by formation of pits on the cell surface.

Morones et al. [29] obtained a similar result although they observed that silver ions gave an additional contribution. They showed that silver nanoparticles act primarily in three ways against Gram-negative bacteria: (1) nanoparticles attach to the surface of the cell membrane and disturb its proper function, like permeability and respiration; (2) they are able to penetrate inside the bacteria and cause further damage by possibly interacting with sulfur- and phosphorus-containing compounds such as DNA; (3) nanoparticles release silver ions, which will give an additional contribution to the bactericidal effect of the silver nanoparticles.

Lock et al. [30] synthesized spherical nano-Ag (average diameter~9.3 nm) particles using a borohydride reduction method and investigated their antibacterial action against *E. coli* by proteomic approaches (2-DE and MS identification). The proteomic data revealed that a short exposure of *E. coli* cells to nano-Ag resulted in an accumulation of envelope protein precursors, indicative of the dissipation of proton motive force. Consistent with these proteomic findings, nano-Ag were shown to destabilize the outer membrane, collapse the plasma membrane potential and deplete the levels of intracellular ATP. The mode of action of nano-Ag was also found to be similar to that of Ag^+ ions [25].

Furthermore the antimicrobial properties of silver nanoparticles were recognized shape and size dependent. Pal et al. [31] investigated the antimicrobial property of differently shaped nanoparticles against *E. coli* both in liquid and agar plate. Truncated triangular silver nanoplates displayed the strongest biocidal action, compared with spherical and rod-shaped nanoparticles. Energy-filtering transmission electron microscopy images revealed considerable changes in the cell membranes upon treatment, resulting in cell death. Elechiguerra et al. [32] found that silver nanoparticles underwent a size-dependent interaction with human immunodeficiency virus type 1, preferably via binding to gp120 glycoprotein knobs. The same group [29]

demonstrated that the only nanoparticles that present a direct interaction with the bacteria preferentially have a diameter of ~1-10 nm.

2.5 Applications

In literature most works investigate silver as antimicrobial additive into various matrices: silver nanoparticles were embedded in a matrix of amorphous silicon dioxide (SiO_2) [33], were impregnated in bacterial cellulose (BC) [34]. Silver ions were added in ZnO nanoparticles [35] and the Mexican zeolitic mineral from Taxco, Guerrero was exchanged with silver ions [36]. Some important advantages of silver-based antimicrobials are their excellent thermal stability and their health and environmental safety.
The mechanism underlying the bactericidal activity of silver loaded materials remains generally unclear. Some reports have suggested that the antibacterial activity of silver loaded materials is realized via the elution of silver ions into the system containing microorganisms, leading to cell death through cell penetration and binding at specific sites to DNA, RNA, respiratory enzymes and cellular protein [37-39]. Others attribute the bactericidal activity of silver loaded materials to catalytic oxidation involving reactive oxygen species (ROS) [40-42].

Medical devices

Resin composites with antibacterial activity may be useful to decrease the frequency of secondary caries around restorations. Yoshida et al. [43] investigated the antibacterial activity of TEGDMA-UDMA-based light-activated resin composites incorporating one of three silver-containing materials (Novaron, Amenitop and AIS) and evaluated the antibacterial activities against

Streptococcus mutans, mechanical properties and release of silver ions. They suggested that the inhibitory effect of three silver-containing materials against *S. mutans* can be attributed to the catalytic action of silver at the surface of the resin composite or to an anti-adhesion property of the surface.

Hospital patients that are mechanically ventilated through intubation with endotracheal tubes are at an increased risk of acquiring pulmonary infections caused by bacteria residing in the hospital. One way to minimize the risk could be to make the surfaces on endotracheal tubes antibacterial with the aim to prevent bacterial adhesion rather than to treat an already formed biofilm. Ramsted et al. [44] deposited silver onto the tube surface through wet chemical treatment and bacterial growth on the treated surface was assayed using *Pseudomonas aeruginosa*. Bacterial growth tests showed that all the modified surfaces inhibited bacterial growth on the tube pieces but also in the surroundings of the tube, probably due to leaching of silver ions.

Implantable devices are major risk factors for hospital-acquired infection. Biomaterials coated with silver oxide or silver alloy have all been used in attempts to reduce infection. Furno et al. [45] developed a new approach using supercritical carbon dioxide to impregnate silicone with nanoparticulate silver metal. They concluded that this impregnation method presented two advantages: the continued release of silver ions in antimicrobial concentrations and the ability to protect both inner and outer surfaces of catheters.

Pyrolytic carbon has been widely used in cardiovascular surgery as artificial heart valves because of its remarkable biocompatibility and mechanical properties. Tang et al. [46] investigated the biocompatibility and bactericidal properties of Ag^+-implanted pyrolytic carbon. The pyrolytic carbon samples were implanted by silver ions with the dose ranging from $5*10^{14}$ to $5*10^{18}$ ions/cm^2 at an energy of 70 keV. They showed that the bactericidal rate for both

S. aureus and *E. coli* increased with the ion dose when the silver ion dose was under the saturated dose of $5*10^{17}$ ions/cm^2. The bactericidal rate was over 97 % when the ion dose exceeded that value. Furthermore Ag^+-implanted pyrolytic carbon showed a good biocompatibility without biotoxication.

Yuranova et al. [47] investigated the fixation of Ag cluster on cotton (cotton-Ag) that would protect wounds from bacterial growth and contamination. For fixation of silver on cotton textile, the precursor solution was prepared by adding isopropanol to the ammonia aqueous solution of $AgNO_3$. The cotton textile was then immersed in this solution and boiled for 1 h. Subsequently, the Ag-cotton sample was removed from the solution, washed with water and sonicated several times before drying at 25 °C. The antimicrobial tests, using *E. coli*, were conducted in the dark and under light. The antimicrobial activity of the cotton was negligible in the dark and increased under solar simulated light. Furthermore an efficient bactericide performance was attained with a very low loading of Ag (0.1 wt.% Ag/wt).

Water treatment

Microfiltration track membranes (TMs) are widely used in separation of virus containing and colloid solutions and in final stages of drinking water treatment under household conditions. When a membrane is brought in contact with an aqueous medium in the course of filtration, various components, with high and low molecular weight, are sorbed on its surface, as also do microbe cells to form a biological film. This film is firmly fixed on the membrane surface, which leads to contamination of drinking water with products formed by microorganisms. Solov'ev et al. [48] developed a method for modification of track membranes by immobilization of polymer complexes of colloid silver nanoparticles on the surface of

microfiltration membranes. Track membranes modified with silver nanoparticles presented antimicrobial effect on *E. coli*, *P. aeruginosa*, *S. aureus* and *B. cereus*.

Activated carbons find widespread use in removing pollutants from gas and liquid phases and in wastewater treatment and water purification processes. One way to foster new applications involves the modification of their surfaces through chemical reactions or through deposition of new materials with attractive properties, such as bactericidal agents. In this respect, silver is an excellent candidate that complies with the bactericidal requirements. Ortiz-Hibarra et al. [49] studied an electrochemical reactor operated under different flow conditions to deposit silver from aqueous $AgNO_3$ solutions and tartaric acid on a commercial activated carbon. The bactericidal activity of the carbon/silver samples was tested on drinking water inoculated with *E. coli*. The XPS results suggested the formation of Ag_2O and AgO surface species and confirm the reduction of silver to the metallic form. Antimicrobial activity toward *E. coli* indicated reductions by up to 7 orders of magnitude in the log CFU/mL in just 10 min contact time and for silver contents of 2.47 wt%. Le-Pape et al. [42] tested, in a dynamic aqueous system, the antimicrobial activity of four activated carbon fibres (ACF), impregnated or not with metallic compounds. All ACF supporting silver exhibited strong lethal activity against *E. coli*, *S. cerevisiae* and *P. pastoris*. Cell mortality was clearly shown by ATP measurements, and was obtained after a very short contact time (a few seconds) between microorganisms and the ACF supporting silver. Maioli et al. [50] tested a commercially available activated carbon filter for water purification enriched with silver. The microbial growth was in general more inhibited in the presence of metal silver into the activated carbon with respect to filters with the activated carbon alone: >4 log inhibition of bacterial proliferation after 78 days of incubation the presence of silver vs. 2 log without silver.

Chang et al. [51] investigated the catalytic inactivation of *Escherichia coli* in water by silver loaded alumina (Ag/Al_2O_3 and $AgCl/Al_2O_3$). They demonstrated that catalyst had a strong catalytic inactivation of *E. coli* at room temperature in water and the fixed silver species and/or eluted Ag^+ strongly enhanced the bactericidal activity of the catalyst by catalyzing the reaction of adsorbed O_2 to ROS (Reactive Oxygen Species) such as H_2O_2, $\cdot OH$ and $\cdot O_2^-$. The catalyst caused the destruction of the cell wall of the bacteria and finally the complete lysis of the bacterial cell. Furthermore they suggested that the catalytically bactericidal effect should be considered as a synergic action of ROS and Ag^+, not as an additive one.

Food packaging

Developing antimicrobial films intended for food packaging applications is one of the emerging research activities in the fields of both applied microbiology and packaging. The active component of these antimicrobial systems can be either organic or inorganic. In particular, the inorganic systems are based on metal ions such as silver, copper, and platinum [52].

An interesting example of an active package based on silver ions is represented by antimicrobial films based on the adhesion of silver zeolite particles on laminate surfaces in contact with foods. This system is widely used as polymer additives for food applications, especially in Japan. Sodium ions present in zeolites are substituted by silver ions. These substituted zeolites are incorporated into polymers like polyethylene, polypropylene, nylon, and butadiene styrene at levels of 1% to 3% [53].

Del Nobile et al. [52] investigated the effectiveness of active films obtained by depositing via plasma an Ag-containing polyethylenoxide-like coating on a polyethylene layer in inhibiting

the growth of *Alicyclobacillus acidoterrestris,* a thermal resistant food spoilage microorganism, in acidic beverages. They suggested that the proposed active film can be successfully used for inhibiting or reducing the microbial growth and also indicated that the effectiveness of the active film depends on the type of medium (Malt Extract Broth or apple juice). Results from silver ion release tests showed that the effectiveness of the proposed active film was strictly related to the amount of silver ion released into the medium.
Tankhiwale and Bajpai [54] demonstrated that grafting of acrylamide onto filter paper, followed by incorporation of silver nanoparticles was a novel biomaterial which showed fair biocidal action against *E. coli*, and so it could be used as an antibacterial packaging material to prevent food stuff from bacterial infection. Furthermore filter paper had a degradable nature.
Silver based antibacterial hybrid materials have been developed after in situ reduction of silver nitrate adsorbed on cellulose fibres [55]. Fibre structure and silver reduction method influenced the morphology of the in situ created silver nanoparticles, and therefore their antimicrobial activity. Hybrid materials were effective against pathogenic microorganisms in vitro and showed very positive results in assays with chicken exudates, both for mesophiles and lactic acid bacteria counts.

2.6 Silver toxicity

The silver toxicity is recognized only in argyria [56, 57] which is due to the excessive use of $AgNO_3$. There are no regular reports of silver allergy [58] and silver toxicity is insignificant compared to the benefits of use and in comparison with other antimicrobials. Sensitization to silver is rarely described and at a lower incidence compared to other topical antimicrobials used in personal care and wound dressings.

In the environment, silver is usually associated with sulfide minerals and released into soil and surface water through weathering. However, in areas where silver levels are high, toxicity to fish and marine life has been noted. Uncontrolled release of silver into the water supply from wastewater treatment plants and accumulation in waste products may have a secondary effect on animals and plants that may have long-term effects e.g. affecting the food chain, so this needs close monitoring [59].

The silver toxicity is related to individual silver species rather than total silver concentration. In natural waters, where silver contamination can be of concern, evidence of toxicity from the dissolved silver ion is generally less than in laboratory tests because of the rich opportunities for possible covalent, complexing, or colloidal binding silver encounters with a variety of reactants. The majority (94%) of the silver released into the environment will remain in the soil or wastewater sludge at the emission site [60].

Silver nanoparticles are typically used in the size range of 1-50 nm. At this very small size, the particles surface area is large comparative to its volume. The comparatively large surface area of nanoparticles increases their reactivity, which in many instances also increases toxicity. Hussain et al. [61] studied the toxicity of different sizes of silver nanoparticles on rat liver cell line (BRL 3A) (ATCC, CRL-1442 immortalized rat liver cells). The authors found that after an exposure of 24 h the mitochondrial cells displayed abnormal size, cellular shrinkage and irregular shape. The biocompatibility and toxicity of Ag nanoparticles were exhibited by observing single Ag NPs inside embryos at each development stage. The types of abnormalities in zebrafish were strongly dependent on the dose of nanoparticles [62].

2.7 Conclusions

Silver is recognized as a good antimicrobial agent. This property is known for silver in the form of ions, colloidal particles, nanoparticles, metallic silver as well as silver compounds and many works study their use to inhibit the proliferation of microorganisms for medical, food packaging and water treatment applications.
Generally silver ions, so as heavy metals, lead to the inactivation of proteins reacting with thiol groups (-SH) on the membrane of bacteria causing the microbial cell death. It has been reported that the attack of silver ions eliminates the replicating ability of DNA and causes the collapse of the cell wall. Furthermore micromolar levels of Ag^+ ions inhibit respiratory chain enzymes and also inactivate the growth of some microorganisms (*Legionella pneumophila*, *Pseudomonas aeruginosa* and *Escherichia coli*) by the chemisorption properties of silver ions onto bacteria.
The antimicrobial properties of silver nanoparticles are shape and size dependent. It was demonstrated that truncated triangular silver displayed the strongest biocidal action, compared with spherical and rod-shaped nanoparticles and also that silver nanoparticles with a diameter of 1-10 nm attached to the surface of cell membrane and after penetrating inside the bacteria caused damage to the DNA.
The possibility to use different materials, treated with silver, as antimicrobial agent is investigated in many works.

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Chapter 3

Antimicrobial Activity

Abstract

In this work we studied the possibility to use thin silver flakes as antimicrobial agent in aqueous medium. The flakes were the waste product of an Italian metallurgic company, thus having zero initial cost. The antimicrobial activity of the flakes was investigated in batch using *Escherichia coli* as Gram-negative microorganism model. The flakes were previously treated with three different activation processes: thermal activation in reducing atmosphere, thermal activation in air and chemical activation. The flakes, activated with either treatment, had antimicrobial activity and acted with different velocity. Furthermore we demonstrated that the antimicrobial capability was amount dependent and the flakes maintained the antibacterial property when reused.

3.1 Introduction

At the beginning of last century, Gibbard [1] was the first to systematically investigate the antimicrobial properties of silver. These properties are known for silver in the form of ions, colloidal particles, nanoparticles, metallic silver as well as silver compounds. Many works study their use to inhibit the proliferation of microorganisms for medical [2], food packaging [3] and water treatment [4, 5] applications. When silver is prepared in the form of very small particles, it is expected to show better antimicrobial characteristics because of their larger specific surface area [6]. With the development of nanotechnology, different works about the bactericidal activity of silver nanoparticles were reported in literature. The antimicrobial properties of silver nanoparticles are shape and size dependent [7]. There are far fewer accounts of the antimicrobial properties of metallic silver [1, 8]. Activation, generally with oxidising agents, is necessary in order to promote the formation and availability of Ag^+ ions. The possibility to use different materials, treated with silver, as antimicrobial agent is investigated in many works [9, 10]. As regards applications of

nanoparticles, they are usually dispersed in a medium or deposited on surfaces in order to perform antimicrobial functions [11, 12]. All these silver-based products show an antimicrobial activity but require complex, time and money consuming production processes (i.e. many chemicals and several steps).
In this work we studied the possibility to use thin silver flakes as antimicrobial agent in aqueous medium. The flakes were the waste product of an Italian metallurgic company, thus having zero initial cost.

3.2 Methods and materials

3.2.1 Flakes features

An Italian company produced thin silver films starting from silver laminates, with a thickness of 10 µm, and using cold beating processes with mechanical hammers. During the cold beating process silver films, with different thickness, were produced. The films obtained in the final cold beating stage were cut, giving a square shape (10 x 10 cm), and inserted in a box. The residues, produced during this step, in the following named untreated flakes (UF), were used in this work.

The UF showed a sub-micrometric dimension: thickness was 300 nm. The UF thickness was measured by a contact stylus profilometer (KLA-TENCOR P10). Because silver flakes reacted electrostatically with charge surface we used a physical vapour deposition silver thin film as holder. In this way the properties of the substrate and the flakes had a good matching. The height and lateral resolution were, respectively, 2 nm and 0.8 µm [13].
The flakes didn't show homogeneous dimension. We determined the dimensional composition of flakes in percentage (table 1) using

sieves with different porosity.

Table 1 - *Dimensional distribution (%) of untreated flakes (UF).*

≥2 mm	850 µm < d < 2 mm	500 µm < d < 850 µm	250 µm < d < 500 µm	150 µm < d < 250 µm	<150 µm
56 %	**23 %**	**10 %**	**7 %**	**3 %**	**1 %**

Furthermore organic impurities present in the flakes were determined by CHN elemental analysis: 0.13 % C, 0.02 % H, 0.01 % N.

3.2.2 Flakes activation processes

The untreated flakes were treated by three different activation processes:

1) Thermal activation in reducing atmosphere (TRA);
2) Thermal activation in air (TA);
3) Chemical activation (CA).

In **TRA** process, UF were heated at 710 °C for 60 seconds in an industrial furnace characterised by reducing atmosphere, which was formed by the ionic dissociation of ammonia (NH_3) at 890 °C. Then the flakes were cooled in the furnace for 3 minutes. The flakes obtained with this process were named reduced flakes (**RF**).

TA process used the same thermal conditions as the TRA process, but it took place in air. The obtained flakes were named thermal flakes (**TF**).

In **CA** process, UF were rinsed, under agitation, in hydrogen

peroxide (H_2O_2) 35 % wt. (Sigma-Aldrich).
The reaction of silver with H_2O_2 is exothermic, producing heat and water vapour. When the reaction stopped the flakes were washed with sterilised deionised water and then were dried in an oven at 105 °C. The flakes obtained were named chemical flakes (**CF**).

3.2.3 Microorganism and culture conditions

The bacterial strain used in this work was Gram-negative *Escherichia coli* (*E. coli* JM109). This strain came from our laboratory collection. *E. coli* is normally used as indicator organism in tests of environmental bacterial contamination. All materials used in the experiments were autoclaved at 121 °C for 30 min to ensure sterility. *E. coli* liquid cultures were prepared in 100 mL of LB medium (yeast extract 5 g/L, tryptone 10 g/L, NaCl 5 g/L) and incubated overnight at 37 °C by constant agitation. A known volume of the liquid culture, chosen on the basis of its initial optical density measured at 600 nm, was centrifuged at 4000 rpm for 15 min. The bacterial pellets were twice washed and resuspended in deionised water. The initial cell density was 10^7-10^9 CFU/mL.

3.2.4 Antimicrobial activity tests in batch

Appropriate amounts of silver flakes (UF, CF, RF, and TF) were added in 50 mL of *E. coli* culture in deionised water medium. The antibacterial activity was investigated in batch at room temperature (22-23 °C), monitoring the cell density by viable cell counts.
At fixed times, the samples were collected and serially diluted in the range of 10^{-1} to 10^{-8}. 100 µL of each dilution were plated on an LB agar plate. The colonies were counted after incubation at 37 °C for 24 h. The reproducibility of the plate counting method was within 0.4 log CFU/mL.

During every test we also monitored the *E. coli* concentration in absence of flakes (control sample). In all the tests, the order of magnitude of the microbe count determined for the control remained unchanged over time.

3.2.5 Determination of Minimum Inhibitory Concentration

Freshly isolated colonies of *E. coli* (JM109) on LB agar were cultured over night in LB broth at 37 °C [14]. After 24 h the cultures were diluted to an optical density at 600 nm of 0.2-0.3 in 10 mL medium. Various masses of the different activated flakes (RF, CF and TF), ranging from 0.001 to 2.931 g, were soaked in 10 mL of culture medium. The mixed media were shaken in the centrifuge tubes for 24 h at 37 °C. The transparency of the medium was measured by visual inspection. When the medium was transparent, we judged that the multiplication of the bacteria was inhibited. The minimum concentration of the flakes in the LB medium (g/L) that maintained the transparency of the medium was defined as the minimum inhibitory concentration (MIC) [15].

3.2.6 Statistical procedure

To verify whether the results of the experiments were significantly different, a statistical procedure for comparing slopes of regression lines was applied at 95 % confidence level [16, pp. 300-302]. The values were considered not different if the statistical test indicated that the null hypothesis H_0 (the values were not different at 95 % confidence level) was accepted; on the contrary, if H_0 was rejected, the values were different.
When the slopes were not significantly different we calculated the common slope (b_{com}) [16, pp. 292-302].

3.3 Results and discussion

3.3.1 Preliminary tests

Preliminary tests were conducted for the development of the analytical methods and to acquire information about the efficiency of the activation processes.

3.3.1.1 Untreated Flakes

The early stage of the research was the study of the untreated flakes antimicrobial activity.
Different amounts of UF (30 mg, 60 mg, 2 g and 4 g) were added to 50 mL of *E. coli* culture in deionised water medium and the bacterial growth was monitored over time. In these experiments the samples were diluted in the range of 10^{-5}-10^{-8}.
In figure 1 the data points obtained in the experiments were reported.
The viable cell count was similar to that in the control even after 24 hours. It was evident that UF hadn't any activity against microorganisms. As we didn't detect a diminished bacterial concentration in solution, this also indicated that the *E. coli* didn't adhere on the flakes surface.
On the basis of these results we treated the flakes with the activation processes, described above, and verified if they acquired the antimicrobial capability.

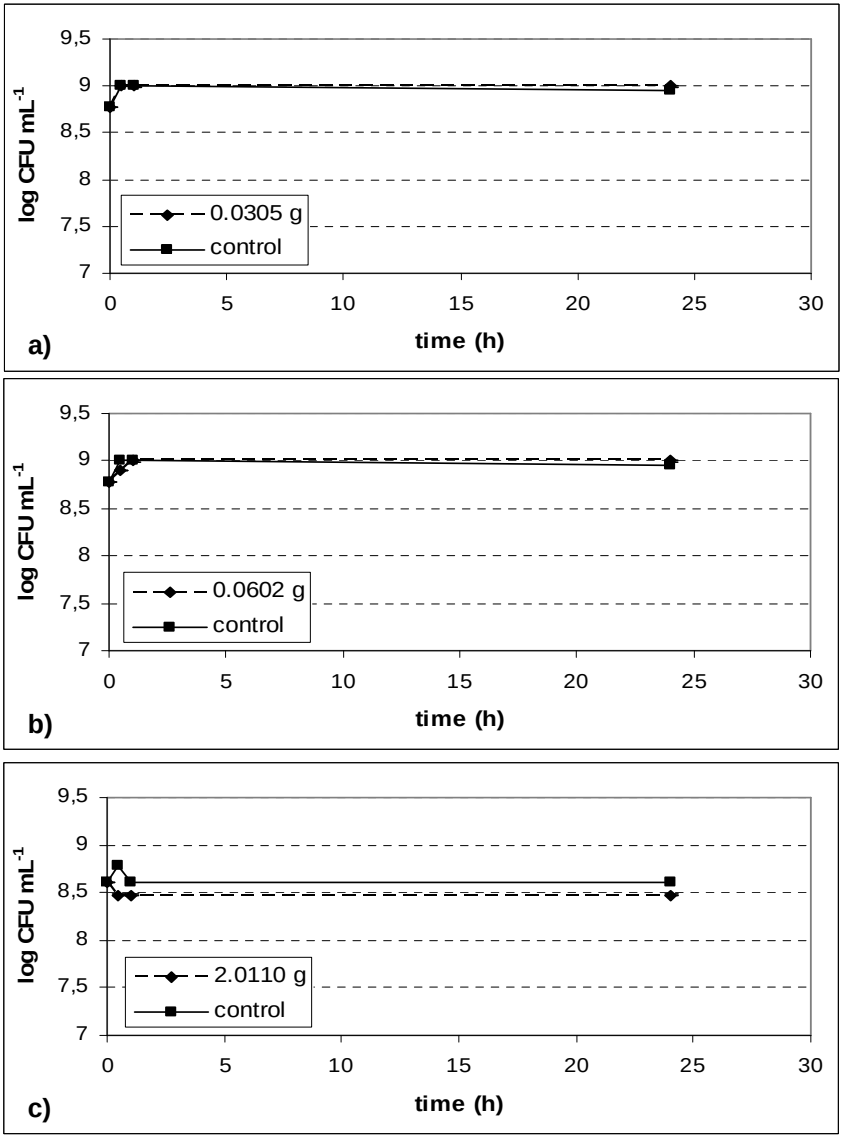


Figure 1(a-f) - *Antimicrobial activity of UF*.

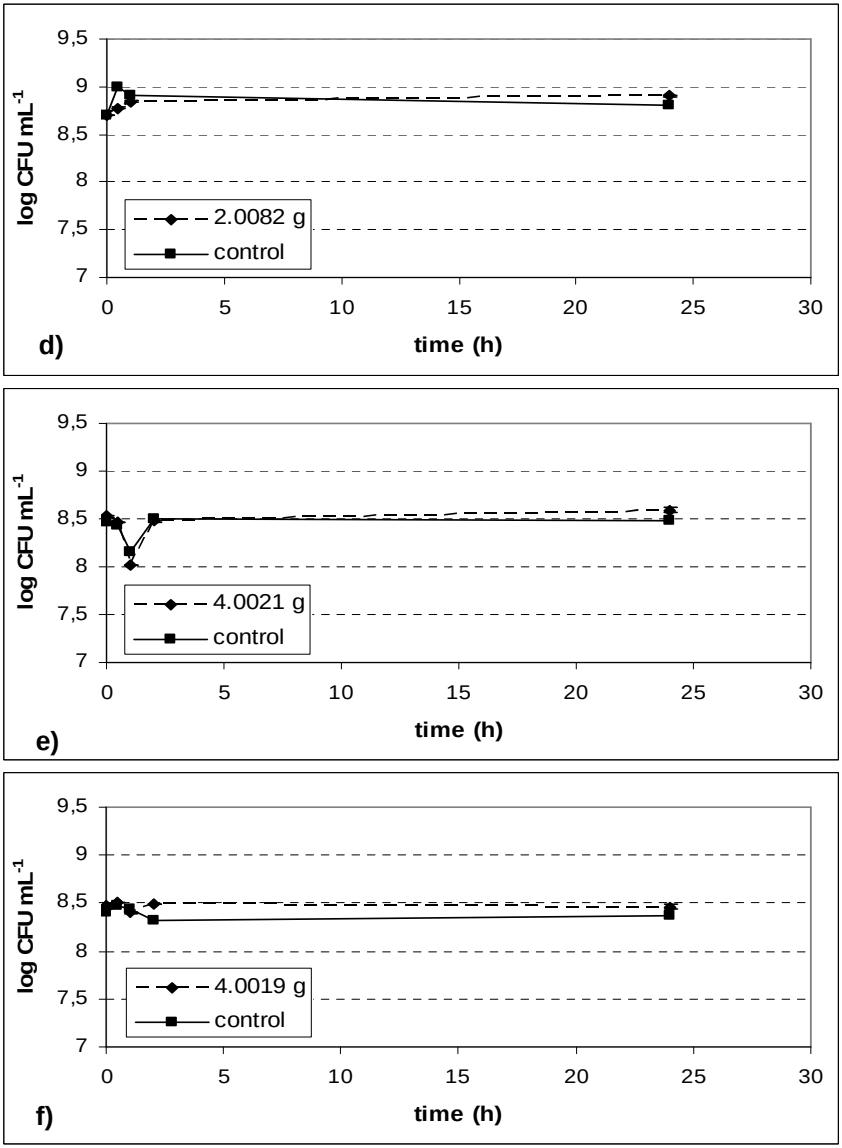


Figure 1(a-f) - *Antimicrobial activity of UF*.

3.3.1.2 Reduced Flakes

We decided to study, at first, the reduced flakes (RF) because the company owned an industrial furnace characterised by reducing atmosphere.
Different amounts of RF (0.5 g, 1 g, 2 g and 4 g) were added to 50 mL of *E. coli* culture in deionised water medium and the bacterial growth was monitored over time. In these experiments the samples were diluted in the range of 10^{-5}-10^{-8}.
The bigger amount of RF tested was 4 g, having a volume coincident with that of the *E. coli* culture. In this way, maximum contact between the microorganism and the flakes was assured.
In figure 2 the data points obtained in the experiments were reported.
The RF showed antimicrobial activity against *Escherichia coli* and it appeared that a longer period of time was required to kill the bacteria, when diminishing RF amount. Furthermore the velocity with which bacteria decreased seemed to be directly proportional to the flakes amount.
Because the results were satisfactory we studied the relationship between kill rate and flakes amount (see par. 3.3.2).

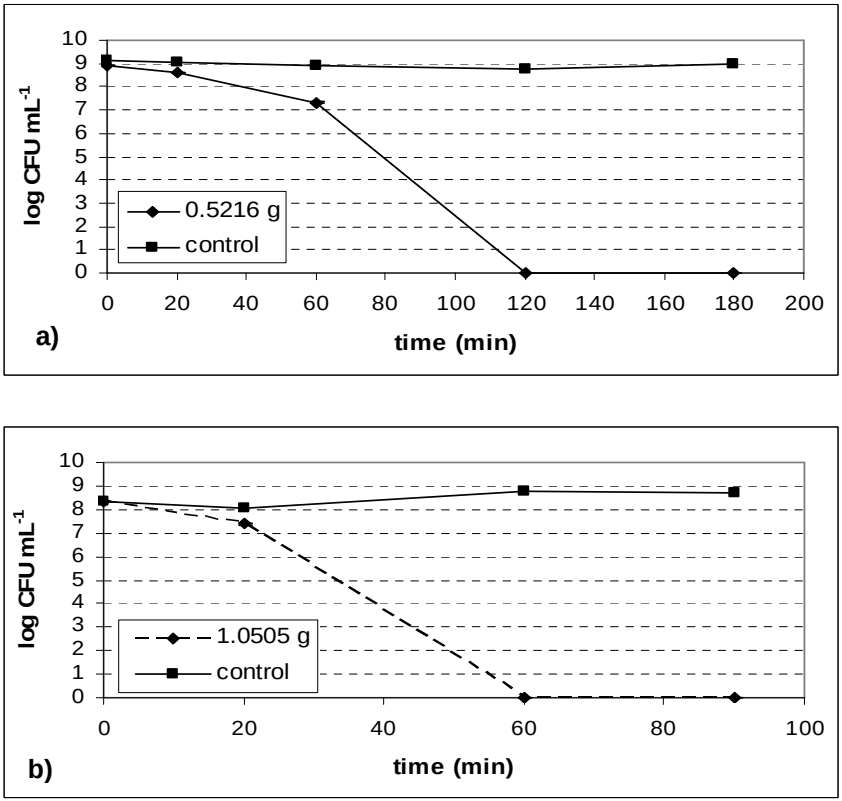


Figure 2(a-d) - *Preliminary tests of RF antimicrobial activity*.

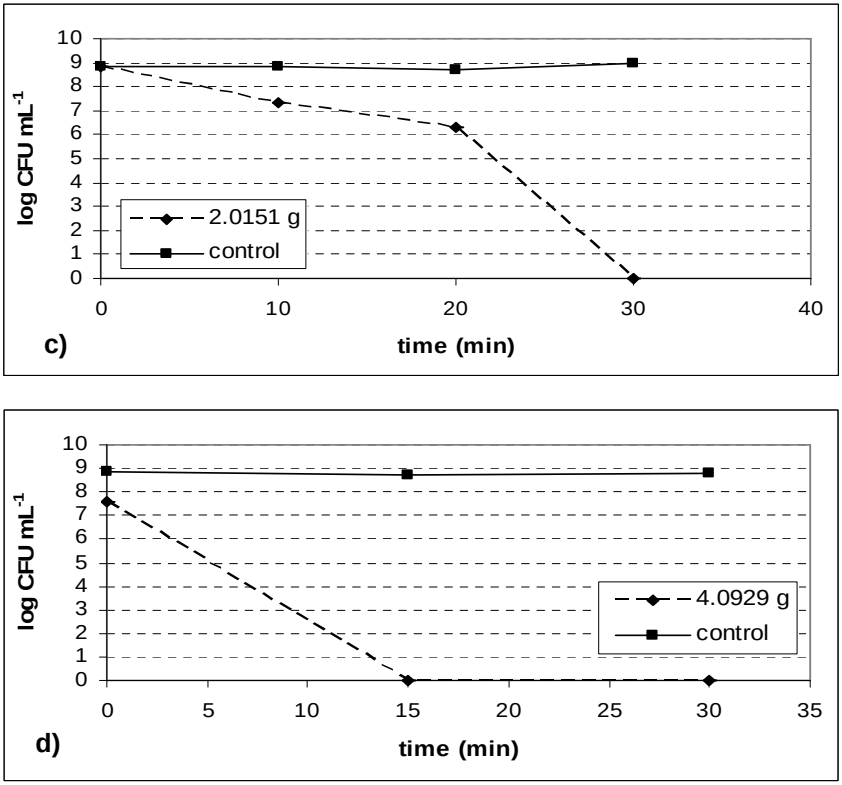


Figure 2(a-d) - *Preliminary tests of RF antimicrobial activity*.

3.3.1.3 Chemical Flakes

As some Authors hypothesised that antimicrobial activity of metallic silver was due to silver oxide [8, 17], we treated silver flakes with the oxidizing agent H_2O_2. 2 g of CF were added to *E. coli* culture in deionised water medium and the bacterial growth was monitored over time. The samples were diluted in the range of 10^{-5}-10^{-8}.
In figure 3 the data points obtained in the experiments were reported.

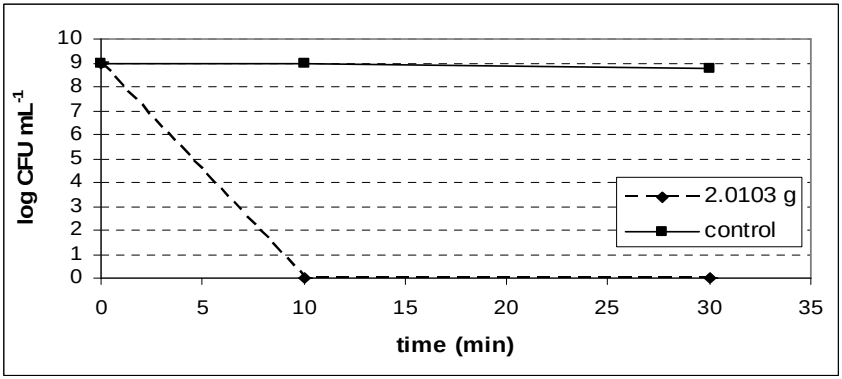


Figure 3 - *Preliminary test of CF antimicrobial activity.*

It seemed that CF possessed a stronger antimicrobial activity than RF; the bacterial cells were killed in ten minutes using only 2 g of CF.
CF used in previous test was washed with deionised water, dried in oven at 105 °C and utilized in a further experiment (figure 4).

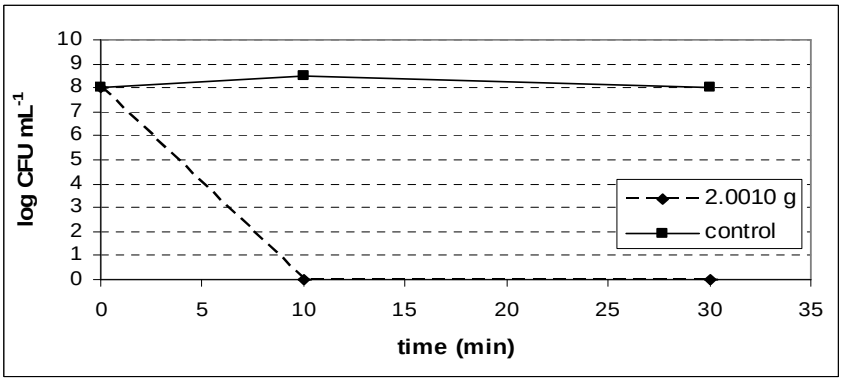


Figure 4 - *Preliminary test of reused-CF (r-CF) antimicrobial activity.*

Apparently CF preserved their antimicrobial activity when reused. So we decided to investigate this property for activated flakes (see par. 3.3.3).

3.3.1.4 Thermal Flakes

4 g of TF were added to *E. coli* culture in deionised water medium and the bacterial growth was monitored over time. The sample was diluted in the range of 10^{-4}-10^{-8}.
In figure 5 the data points obtained in the experiment were reported.

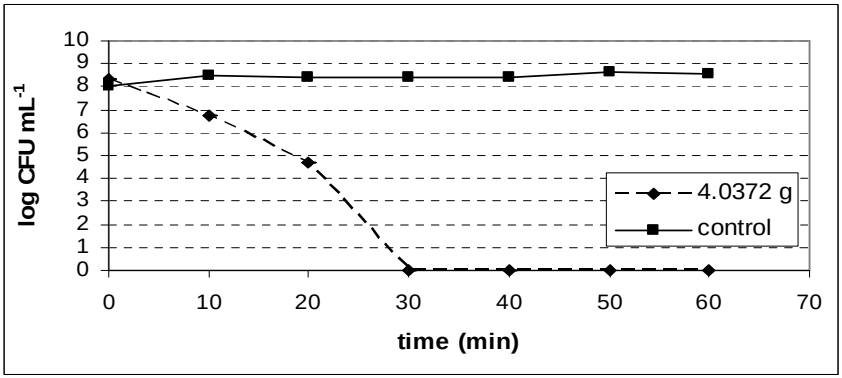


Figure 5 - *Preliminary test of TF antimicrobial activity*.

Also TF presented antimicrobial capability but it was weaker than RF and CF.
We concluded that flakes, by the three different activation processes, could be used as antimicrobial agents. So we decided to examine closely this possible application.
We investigated different antimicrobial activity aspects for the treated flakes. Mainly, using RF, we studied the dependence of the antibacterial activity on the flakes amount; using CF and TF we analyzed the maintenance of the antimicrobial capability.

3.3.2 Flakes amount dependence

Different amounts of reduced flakes (0.05 g, 0.5 g, 1 g, 2 g and 4 g) were added to 50 mL of *E. coli* culture in deionised water and the cells growth was monitored over time. In these experiments the samples were diluted in the range of 10^{-1}-10^{-7}.
In figure 6 the data points obtained using 0.05 g were reported.

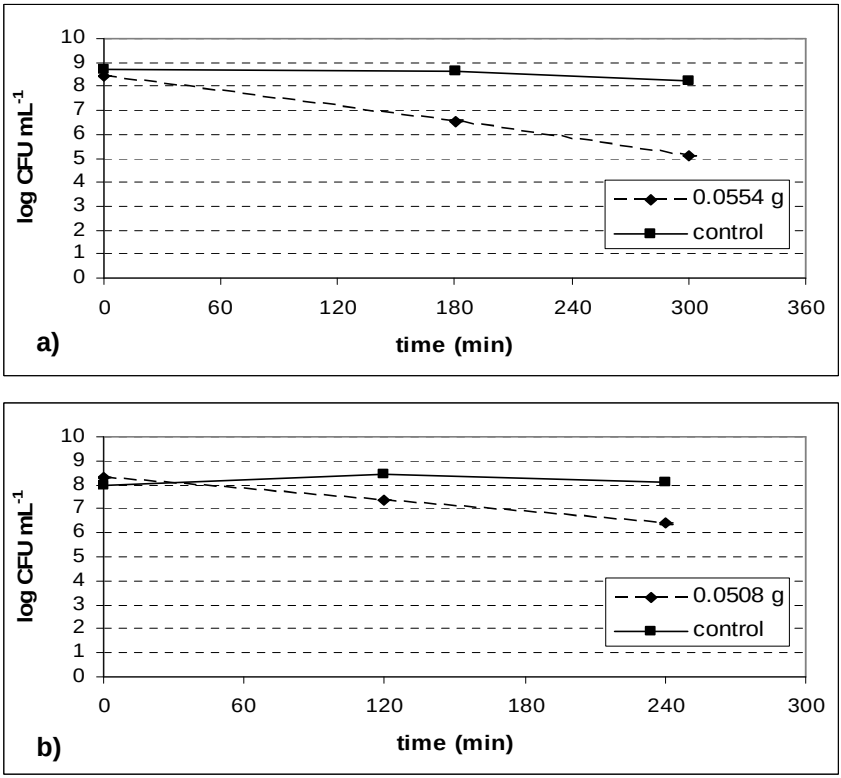


Figure 6(a-c) – *RF antimicrobial activity (0.05 g).*

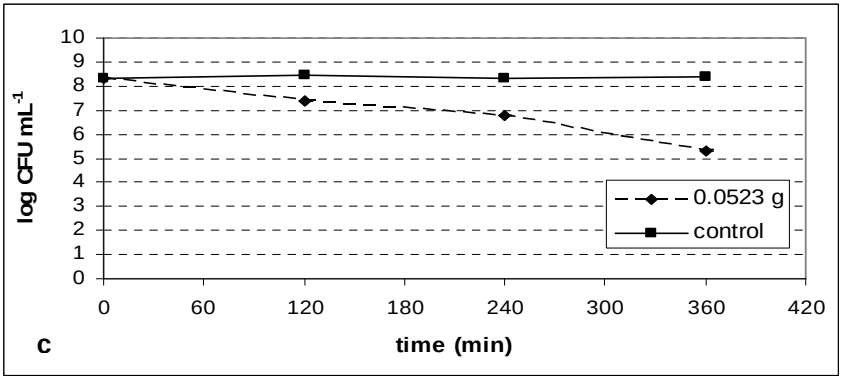
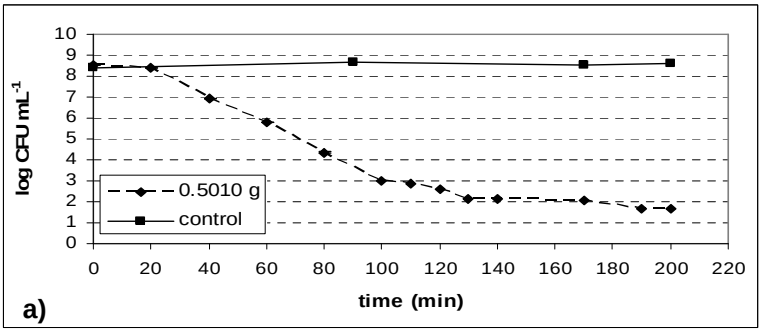
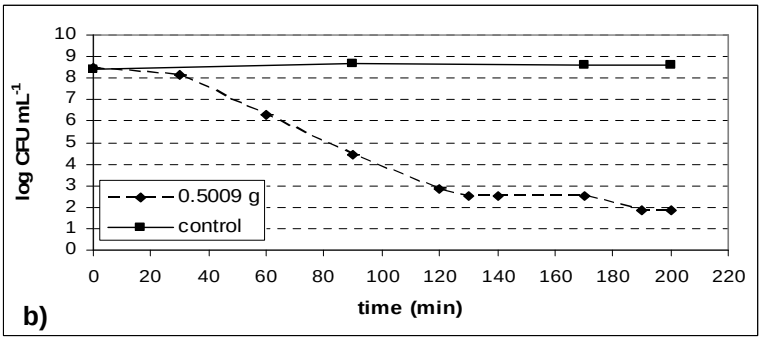


Figure 6(a-c) – *RF antimicrobial activity (0.05 g).*

In figure 7 the data points for the experiments conducted with 0.5 g were reported.





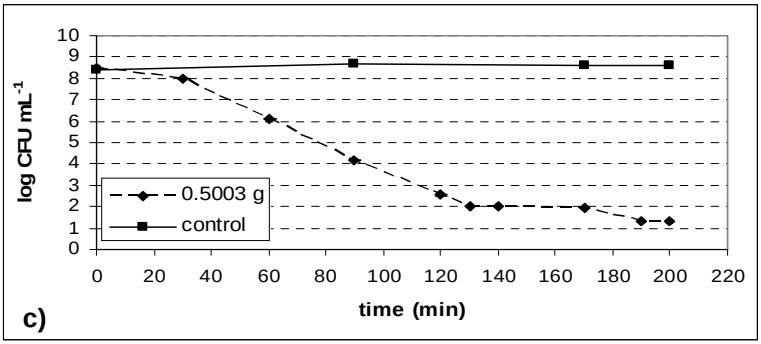


Figure 7(a-c) – *RF antimicrobial activity (0.5 g).*

In figure 8 the data points experiments obtained using 1 g were reported.

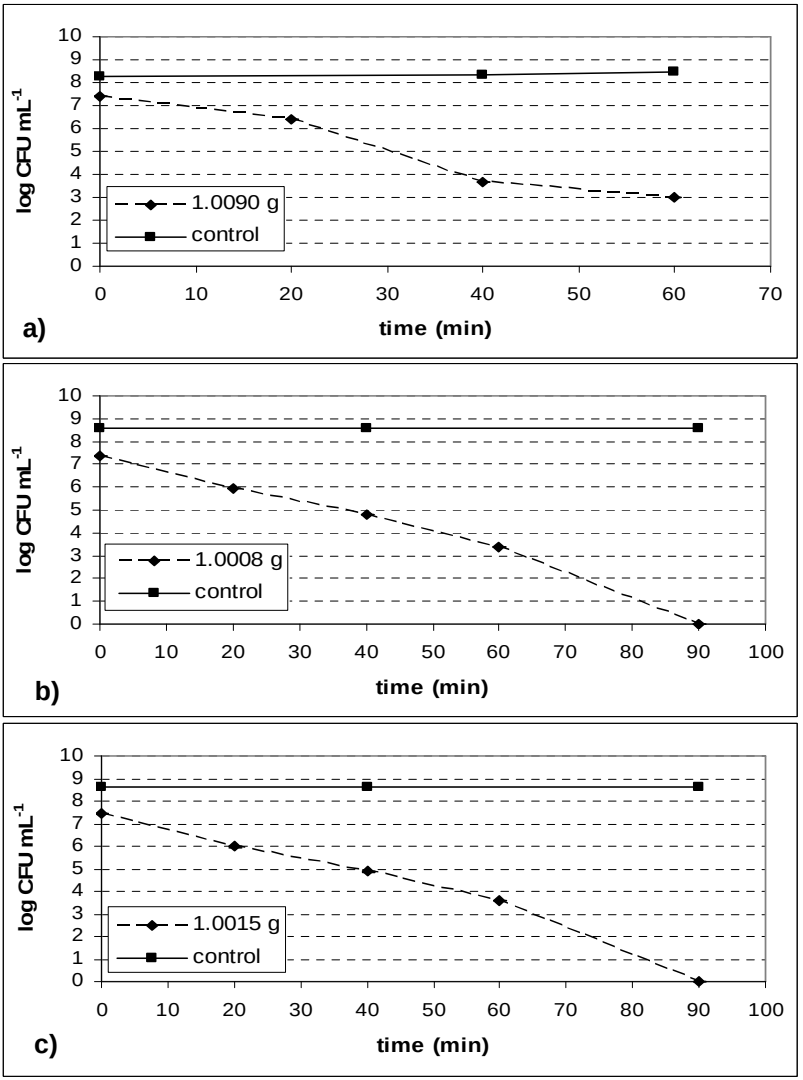


Figure 8(a-c) – *RF antimicrobial activity (1 g).*

In figure 9 the data points experiments using 2 g were reported.

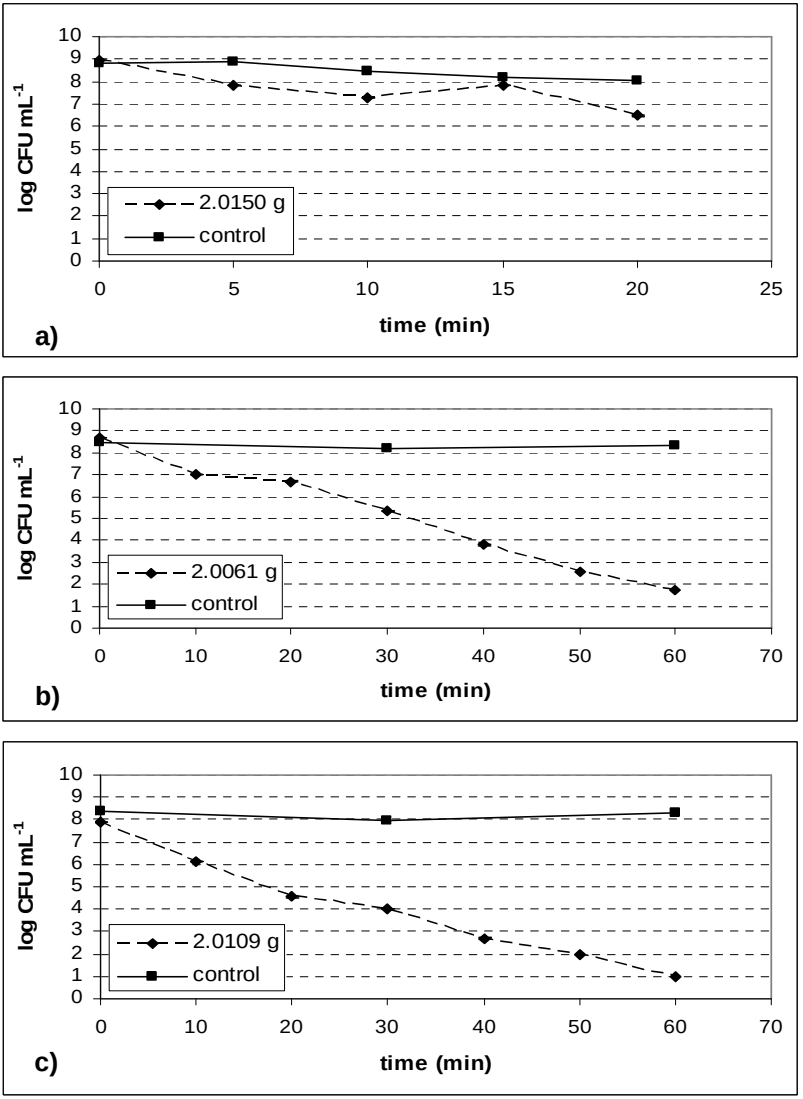


Figure 9(a-c) – *RF antimicrobial activity (2 g).*

In figure 10 the data points obtained using 4 g of RF were reported.

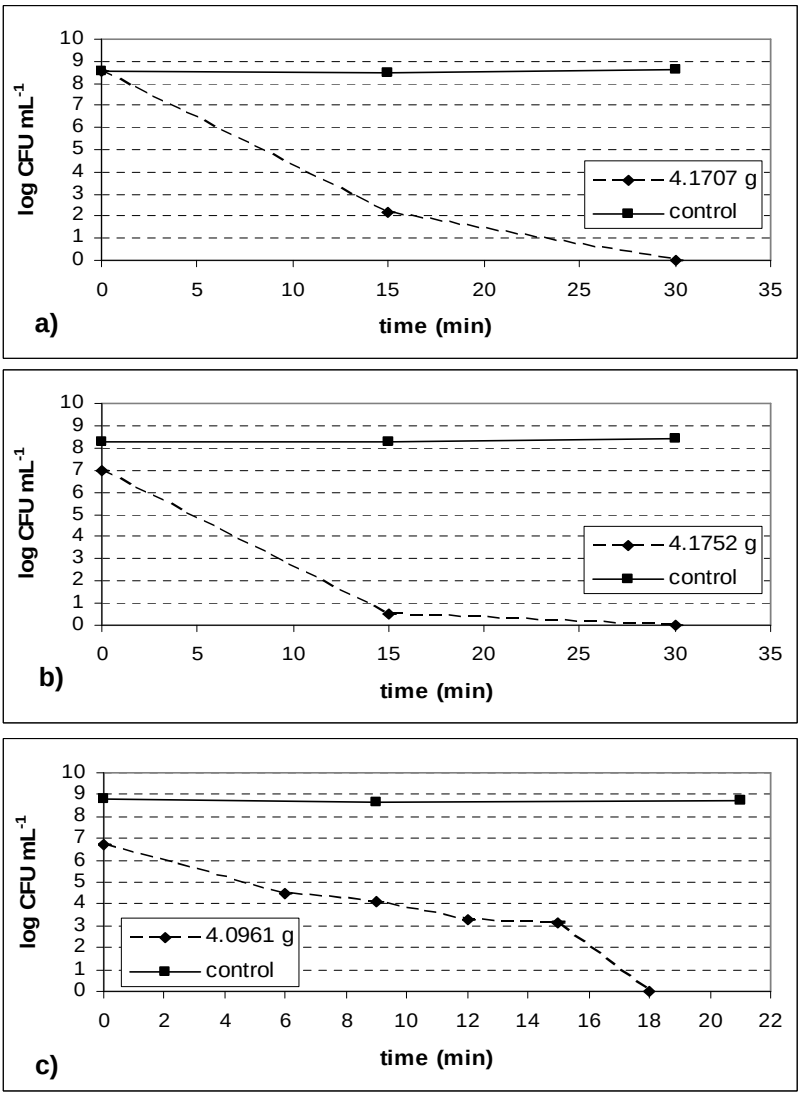


Figure 10(a-f) – *RF antimicrobial activity (4 g).*

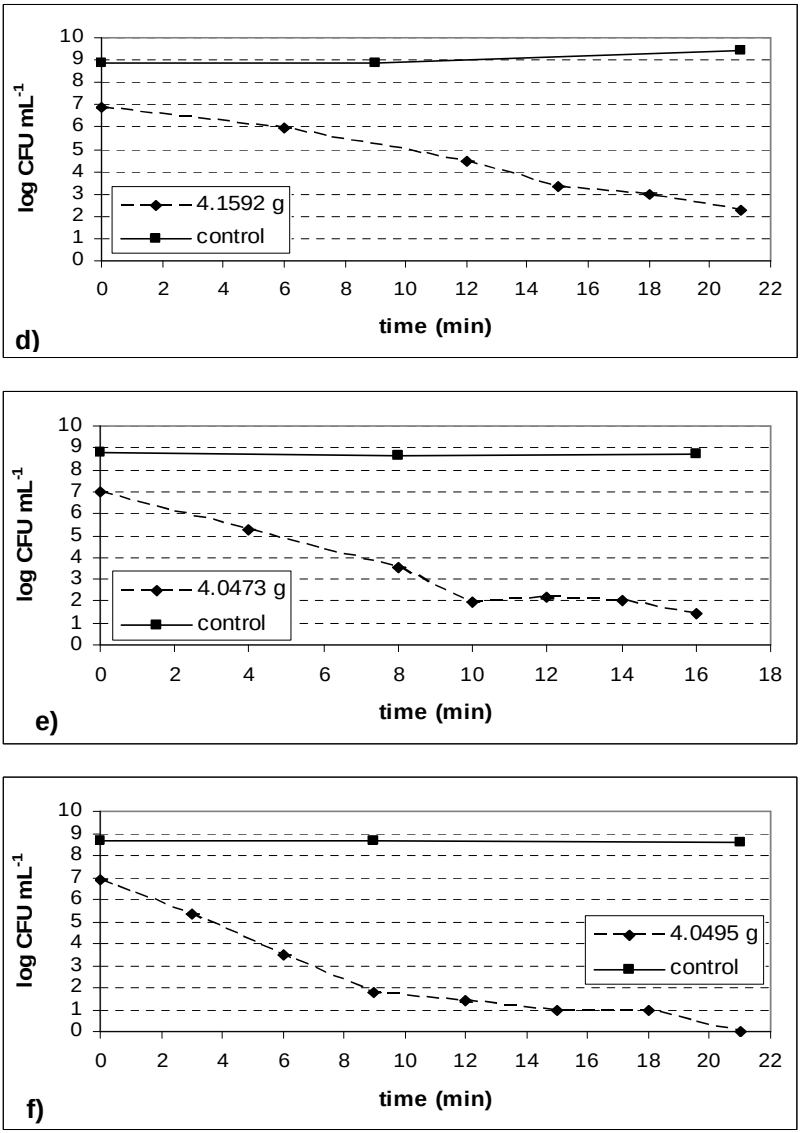


Figure 10(a-f) – *RF antimicrobial activity (4 g).*

We observed that the decrease of *E. coli* concentration with time was faster when the RF amount increased.
With the aim to verify if the experimental data showed a linear trend, we calculated the regression lines and R^2 values for each experiment (table 2). The regression lines were calculated considering as final time the first data point with zero cell concentration.

Table 2 – *Regression line equations and correlation coefficients for each experiments*.

	RF amount (g)	Equation	R^2
RF *(6a)*	0.0554	Y = (-0.011±0.00005)x + (8.46±0.09)	0.9983
RF *(6b)*	0.0508	Y = (-0.0080±0.0001)x + (8.31±0.02)	0.9998
RF *(6c)*	0.0523	Y = (-0.008±0.001)x + (8.4±0.2)	0.9704
RF *(7a)*	0.5010	Y = (-0.038±0.004)x + (8.0±0.5)	0.8857
RF *(7b)*	0.5009	Y = (-0.037±0.004)x + (8.3±0.5)	0.9156
RF *(7c)*	0.5003	Y = (-0.039±0.004)x + (8.2±0.5)	0.9220
RF *(8a)*	1.0090	Y = (-0.08±0.01)x + (7.5±0.5)	0.9422
RF *(8b)*	1.0008	Y = (-0.080±0.007)x + (7.7±0.4)	0.9791
RF *(8c)*	1.0015	Y = (-0.080±0.008)x + (7.7±0.4)	0.9696
RF *(9a)*	2.0150	Y = (-0.10±0.03)x + (8.7±0.4)	0.7626
RF *(9b)*	2.0361	Y = (-0.116±0.006)x + (8.6±0.2)	0.9866
RF *(9c)*	2.0109	Y = (-0.110±0.008)x + (7.3±0.3)	0.9761
RF *(10a)*	4.1707	Y = (-0.28±0.08)x + (7.9±1.5)	0.9270
RF *(10b)*	4.1752	Y = (-0.3±0.1)x + (7.1±2.7)	0.7930
RF *(10c)*	4.0961	Y = (-0.32±0.06)x + (6.8±0.7)	0.8879
RF *(10d)*	4.1592	Y = (-0.23±0.01)x + (7.1±0.2)	0.9864
RF *(10e)*	4.0473	Y = (-0.35±0.04)x + (6.6±0.5)	0.9266
RF *(10f)*	4.0495	Y = (-0.31±0.05)x + (5.9±0.6)	0.8868

The correlation coefficients (R^2) were comprised between 0.89 and 0.99 except for two values (RF *10b* and RF *9a*). On the basis of the regression lines and R^2 values we concluded that the experimental data showed a linear trend for all experiments. Thus, we hypothesized that the decrease of *E. coli* concentration with time followed a first order kinetics.

The slope of each regression line represented the decrease of viable cells, expressed as CFU/mL, in a minute and was termed kill rate [log CFU mL^{-1} min^{-1}].
The slopes of the regression lines determined in the experiments conducted using the same RF amount were compared applying the statistical test at 95 % confidence level. We verified that the slopes were not significantly different and calculated the common slope for each experiments group (table 3) [16, pp. 300-302].

Table 3 – *Common slopes and errors for experiments with different RF amount.*

	Common slope [log CFU mL^{-1} min^{-1}]	Common slope error
$RF_{0.05}$	0.009	±0.001
$RF_{0.5}$	0.038	±0.002
RF_{1}	0.080	±0.006
RF_{2}	0.113	±0.006
RF_{4}	0.30	±0.03

The obtained common slopes were used to analyse the dependence of the rate of bacterial kill on RF amounts.
In figure 11 the relationship between the removal rate and the amount was reported. The data were very well fitted with a linear function, whose equation was also reported in figure 11 with the R^2 coefficient. The intercept of the regression line wasn't significantly different from zero (*t*-test at 95 % significance level), as expected, since we didn't detect any antimicrobial activity in absence of RF.

We concluded that the antimicrobial activity was amount dependent.
It was evident that the killing rate increased with the RF amount with direct proportionality.
Using the kill rates reported in table 3, we estimated the time necessary to have zero CFU mL^{-1} using 0.05 g, 1 g, 2 g and 4 g of RF

and supposing to have an initial cell concentration of 10^8 CFU mL^{-1}. The estimated times were 889 min for 0.05 g, 211 min for 0.5 g, 100 min for 1 g, 71 min for 2 g and 27 min for 4g.

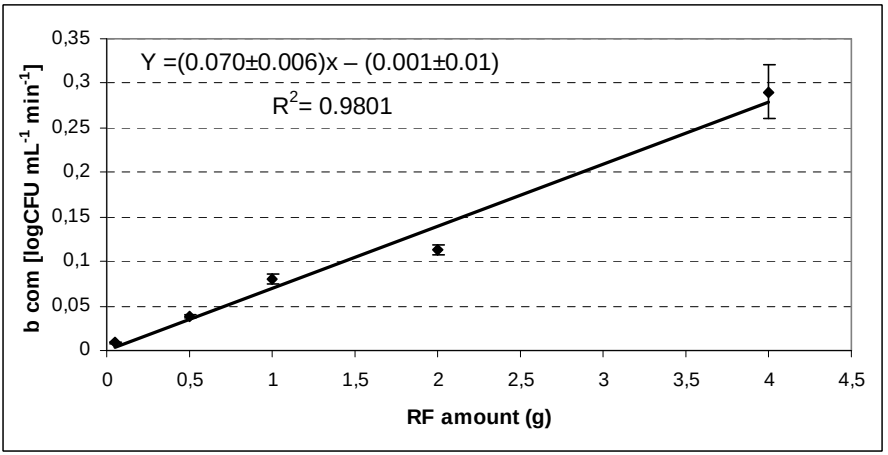


Figure 11 - *Dependence of the removal rate on the amount of RF.*

3.3.3 Maintenance of the antimicrobial capability

1 g of CF were added to *E. coli* culture in deionised water and the bacterial growth was monitored over time. In these experiments the samples were diluted in the range of 10^{-1}-10^{-7}.
Figure 12 showed the data points obtained in experiments for CF.

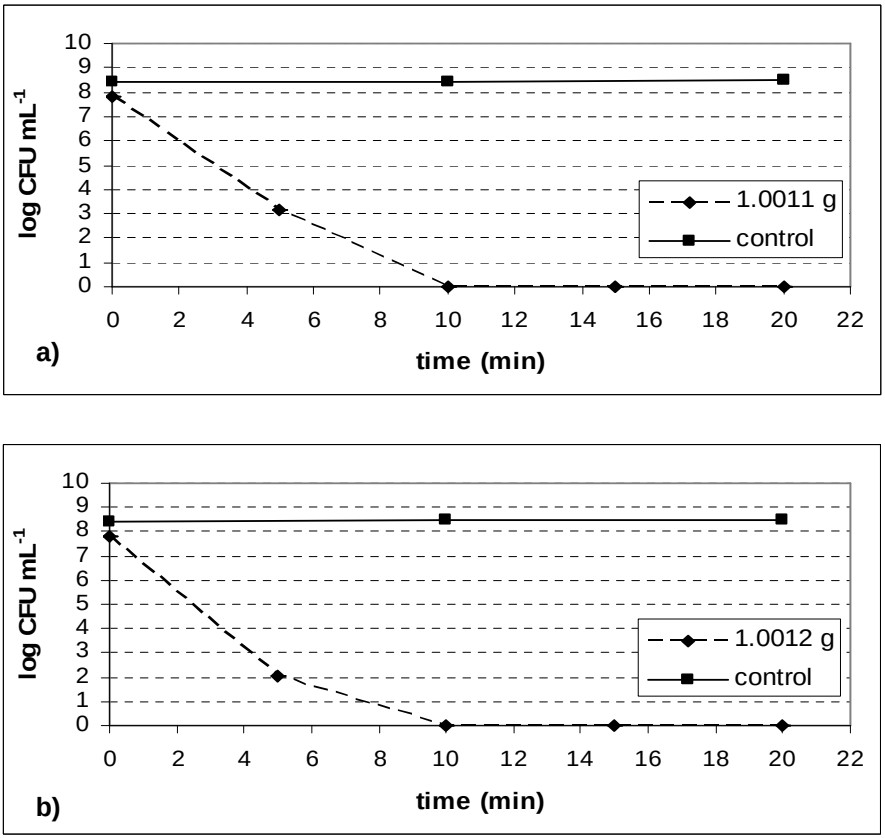


Figure 12(a-d) – *CF antimicrobial activity (1 g).*

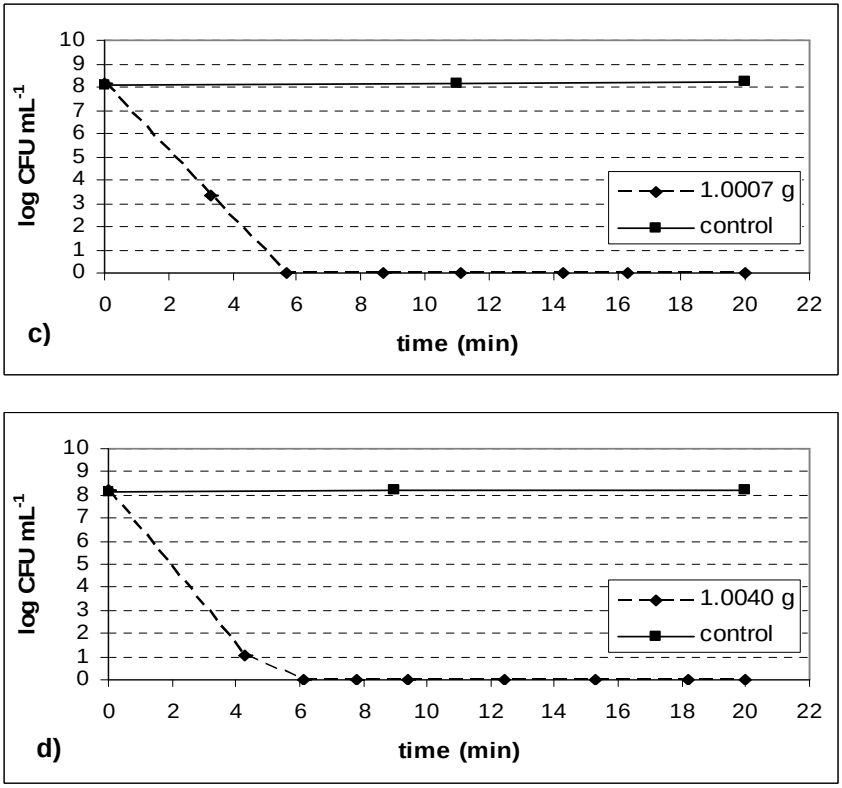


Figure 12(a-d) – *CF antimicrobial activity (1 g).*

With the aim to use the flakes as antimicrobial agent in water treatment, we studied if the reused-CF (r-CF) maintained the antimicrobial capability (figure 13).

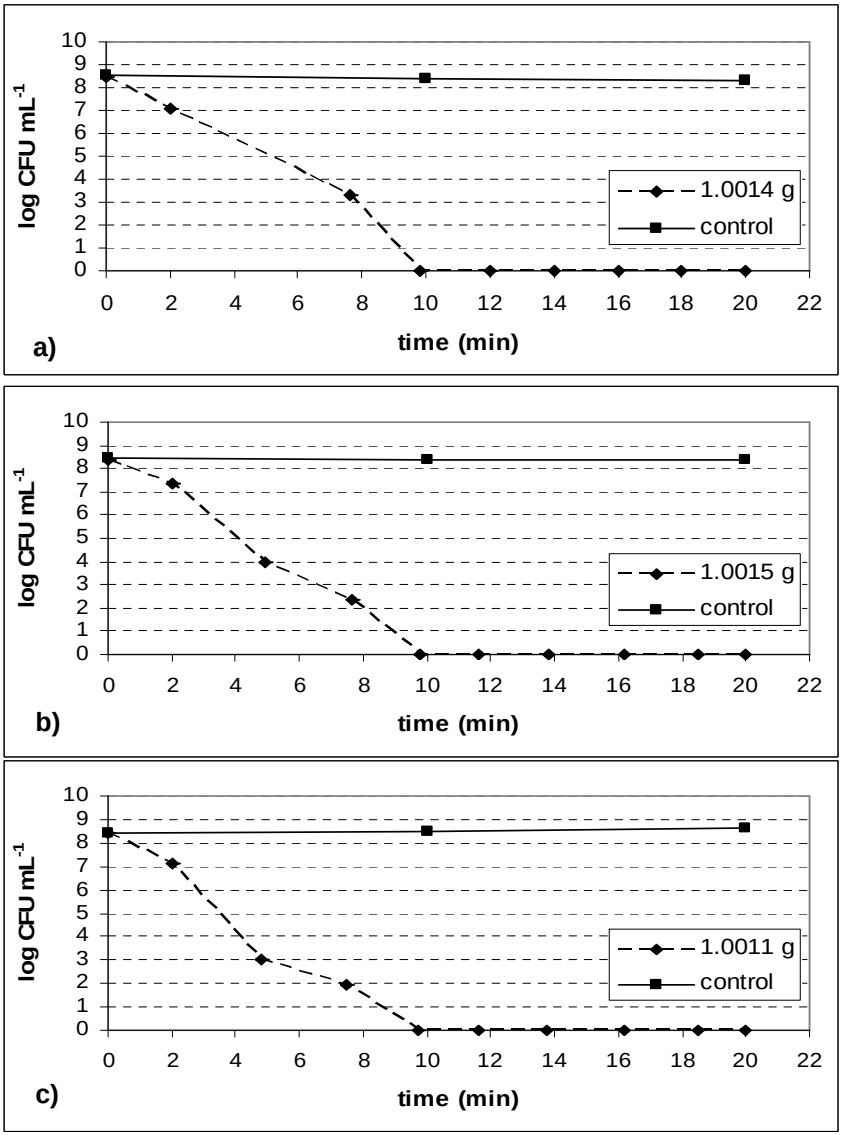


Figure 13(a-c) – *r-CF antimicrobial activity (1 g).*

4 g of TF were added to *E. coli* culture in deionised water and the bacterial growth was monitored over time. In this experiment the samples were diluted in the range of 10^{-1}-10^{-7}.
Figure 14 showed the data points obtained in the experiment for TF.

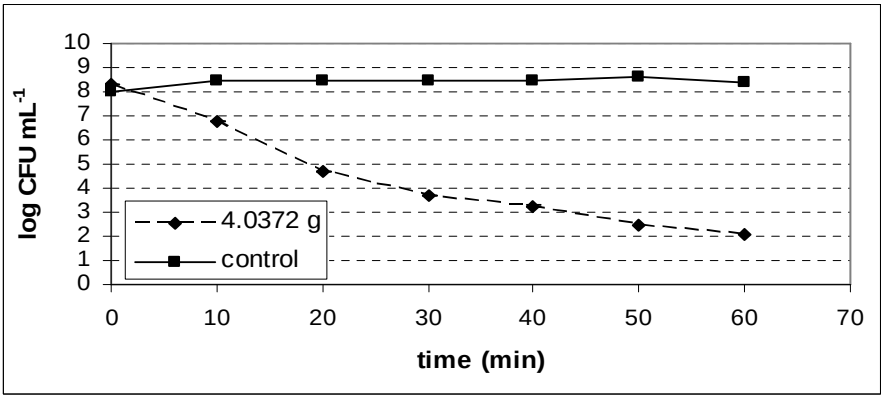


Figure 14 – *TF antimicrobial activity (4 g).*

With the aim to use the flakes as antimicrobial agent in water treatment, we studied if the reused-TF (r-TF) maintained the antimicrobial capability (figure 15).

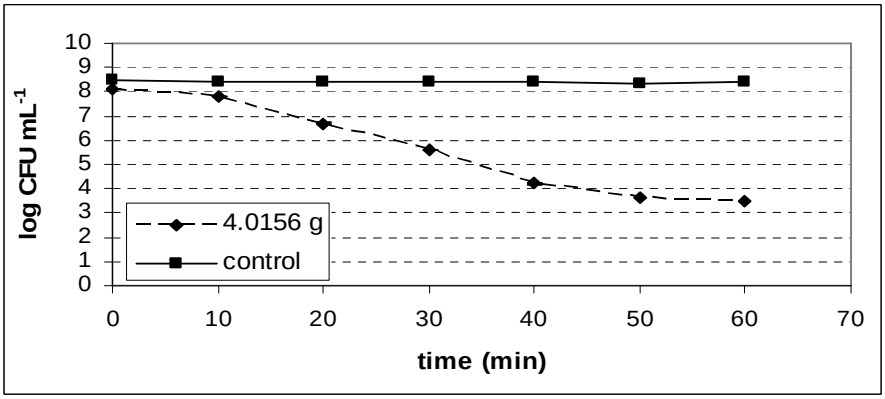


Figure 15 – *r-TF antimicrobial activity (4 g)..*

It was evident that CF and TF maintained the antimicrobial activity when reused, even if apparently a longer time was necessary to completely kill the microorganisms.
We calculated the regression lines and R^2 values for each experiment (table 4). The regression lines were calculated considering as final time the first data point with zero cell concentration.

Table 4 – *Regression line equations and correlation coefficients*.

	Flakes Amount (g)	Equation	R^2
CF *(13a)*	1.0011	Y = (-0.78±0.09)x + (7.6±0.6)	0.9873
CF *(13b)*	1.0012	Y = (-0.8±0.2) + (7±1)	0.9290
CF *(13c)*	1.0007	Y = (-1.44± 0.03) + (8.1±0.1)	0.9996
CF *(13d)*	1.0040	Y = (-1.4± 0.2) + (8±1)	0.9699
r-CF *(14a)*	1.0014	Y = (-0.8±0.1) + (8.7±0.6)	0.9701
r-CF *(14b)*	1.0015	Y = (-0.87±0.06) + (8.6±0.3)	0.9873
r-CF *(14c)*	1.0011	Y = (-0.88±0.09) + (8.4±0.6)	0.9679
TF (15)	4.0372	Y = (-0.10±0.01) + (7.5±0.5)	0.9248
r-TF (16)	4.0156	Y = (-0.089±0.008) + (8.3±0.3)	0.9641

The correlation coefficients (R^2) were greater than 0.9. The slope of each regression line represented the decrease of viable cells, expressed as CFU/mL, in a minute and was termed kill rate [log CFU mL^{-1} min^{-1}].
The slopes were compared applying the statistical tests at 95 % confidence level [16, pp. 300-302]. We demonstrated that the slopes of the regression lines of the experiments conducted with CF and r-CF [16, pp. 300-302], and with TF and r-TF [16, pp. 292-295] were not significantly different.
We calculated the common slope for CF and r-CF experiments (b_{com}= 0.9±0.1 log CFU mL^{-1} min^{-1}) [16, pp. 300-302] and the common slope for TF and r-TF experiments (b_{com}= 0.095) [13, pp. 292-295]. The b_{com} represented the rate of removal of viable cells

using flakes or reused flakes indifferently.
We concluded that the *E. coli* kill rate was constant even if CF and TF were reused.

3.3.4 Minimum Inhibitory Concentration values

Table 5 showed the MIC values against *E. coli* for the flakes treated with the different activation processes.

Table 5 – *MIC values of the activated flakes*.

	MIC Value (g/L)
Reduced Flakes	**80**
Thermal Flakes	**205**
Chemical Flakes	**10**

The MIC value of the chemical flakes was greater than the MIC values of the reduced and thermal flakes. These results were in accordance with the antimicrobial tests where the chemical flakes were the more efficacy.

3.4 Conclusions

The recovery of a waste product from a thin metallic silver films manufacturing company was proposed using it as antimicrobial agent. The silver flakes were treated by thermal activation in reducing atmosphere, thermal activation in air and chemical activation. The flakes obtained by the thermal activation process in reducing atmosphere were named reduced flakes (RF); the flakes obtained by the thermal activation process in air were named thermal flakes (TF) and the flakes obtained by the chemical activation process were named chemical flakes (CF). *E. coli* was used as indicator organism in the antimicrobial tests in deionised water. The activated flakes (RF, TF and CF) acquired antimicrobial properties.
The kill rate of *E. coli* was dependent on the type of activated flakes. The chemical flakes were the more efficient, in fact the kill rate calculated for 1 g of CF (0.9 ± 0.1 log CFU mL^{-1} min^{-1}) was greater than the kill rates calculated for 4 g of RF (0.30 ± 0.03 log CFU mL^{1} min^{-1}) and of TF (0.10 ± 0.01 log CFU mL^{-1} min^{-1}). This was confirmed also by the MIC values.
The antimicrobial capability was dependent on flakes amount. Increasing the flakes amount, the removal rate increased. Furthermore the flakes maintained their properties also when re-used. On the basis of these results it was proposed to use the activated flakes when it was necessary to hinder the microorganisms proliferation.

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Chapter 4

Activated Silver Flakes: Bacteriostatic or Bactericidal?

Abstract

In this work we investigated the activated flakes effect on bacterial cells. The flakes are the waste product of an Italian metallurgic company and they acquired antimicrobial activity with two different activation processes: thermal activation in reducing atmosphere and chemical activation. We observed the *Escherichia coli* treated with activated flakes by fluorescence microscopy and scanning electron microscopy (SEM).
We demonstrated that activated flakes caused cells damage and then their death. We concluded that activated flakes could be considered a bactericidal agent.

4.1 Introduction

The exact mechanism of action of silver on the microbes is still not known but possible mechanisms of action have been suggested according to the morphological and structural changes found in the bacterial cells.
Generally silver ions, so as heavy metals, lead to the inactivation of proteins reacting with thiol groups (-SH) on the membrane of bacteria causing the microbial cell death [1-3]. It has been reported that the attack of silver ions eliminates the replicating ability of DNA and causes the collapse of the cell wall [4]. Furthermore micromolar levels of Ag^+ ions inhibit respiratory chain enzymes [5-6] and also inactivate the growth of some microorganisms by the chemisorption properties of silver ions onto bacteria [7]. The antimicrobial properties of silver nanoparticles are shape and size dependent. It was demonstrated that truncated triangular silver displayed the strongest biocidal action, compared with spherical and rod-shaped nanoparticles [8] and also that silver nanoparticles with a diameter of

1-10 nm attached to the surface of cell membrane and after penetrating inside the bacteria caused damage to the DNA [9].
The antibacterial activity of silver loaded materials can be due to the elution of silver ions into the system containing microorganisms [10] or/and to catalytic oxidation involving reactive oxygen species (ROS) [11].
Various techniques were used to study the mechanism of action and so the effect of silver on bacterial cells. Chen et al. [12] used scanning electron microscopy (SEM) to study the effect of Ag/Al_2O_3 on *Escherichia coli*. Chang et al. [13] studied the effect of silver loaded alumina on *Escherichia coli* using transmission electron microscopy (TEM). They observed that the cells showed dramatic morphological changes. The cells swelled to a much larger size than the untreated ones. Furthermore they released the intracellular ingredients. Feng et al. [4] studied the effect of silver ions on *Escherichia coli* using TEM and observed that Ag^+ ions were detected inside the cells indicating the interaction with thiol groups in cytoplasmic proteins.
Furthermore the membrane integrity of the bacteria, reflected by the influx of membrane-impermeable fluorescent PI, can be used to judge whether the bacteria are still alive [14].
We are investigating the possibility to use activated silver flakes as antimicrobial agent in aqueous medium. In this work we studied the mode of action of flakes using two techniques: fluorescence microscopy and scanning electron microscopy (SEM).
We showed that *E. coli* cells were damaged when treated with activated flakes: 10-20 % of the cells appeared damaged and 80-90 % dead at fluorescence microscopy observation. SEM photographs showed cells disruption and consequently release of the intracellular constituents.

4.2 Methods and materials

4.2.1 Flakes features

An Italian company produced thin silver films starting from silver laminates, with a thickness of 10 µm, and using cold beating processes with mechanical hammers. During the cold beating process silver films, with different thickness, were produced. The films obtained in the final cold beating stage were cut, giving a square shape (10 x 10 cm), and inserted in a box. The residues, produced during this step, in the following named untreated flakes (UF), were used in this work.

The UF showed a sub-micrometric dimension: thickness was 300 nm. The UF thickness was measured by a contact stylus profilometer (KLA-TENCOR P10). Because silver flakes reacted electrostatically with charge surface we used a physical vapour deposition silver thin film as holder. In this way the properties of the substrate and the flakes had a good matching. The height and lateral resolution were, respectively, 2 nm and 0.8 µm [8].

The flakes didn't show homogeneous dimension. We determined the dimensional composition of flakes in percentage (table 1) using sieves with different porosity.

Table 1 - D*imensional distribution (%) of untreated flakes (UF).*

≥2 mm	850 µm < d < 2 mm	500 µm < d < 850 µm	250 µm < d < 500 µm	150 µm < d < 250 µm	<150 µm
56 %	**23 %**	**10 %**	**7 %**	**3 %**	**1 %**

Furthermore organic impurities present in the flakes were determined by CHN elemental analysis: 0.13 % C, 0.02 % H, 0.01 % N.

4.2.2 Flakes activation processes

The untreated flakes were treated by two different activation processes:

1) Thermal activation in reducing atmosphere (TRA);
2) Chemical activation in air (CA).

In **TRA** process, UF were heated at 710 °C for 60 seconds in an industrial furnace characterised by reducing atmosphere, which was formed by the ionic dissociation of ammonia (NH_3) at 890 °C. Then the flakes were cooled in the furnace for 3 minutes. The flakes obtained with this process were named reduced flakes (**RF**).

In **CA** process, UF were rinsed, under agitation, in hydrogen peroxide (H_2O_2) 35 % wt. (Sigma-Aldrich).
The reaction of silver with H_2O_2 is exothermic, producing warm and water vapour. When the reaction stopped the flakes were washed with sterilised deionised water and then were dried in an oven at 105 °C. The flakes obtained were named chemical flakes (**CF**).

4.2.3 Microorganism and culture conditions

The bacterial strain used in this work was Gram-negative *Escherichia coli* (*E. coli* JM109). This strain came from our laboratory collection. *E. coli* is normally used as indicator organism in tests of environmental bacterial contamination. All materials used in the experiments were autoclaved at 121 °C for 30 min to ensure sterility. *E. coli* liquid cultures were prepared in 100 mL of LB

medium (yeast extract 5 g/L, tryptone 10 g/L, NaCl 5 g/L) and incubated overnight at 37 °C by constant agitation. A known volume of the liquid culture, chosen on the basis of its initial optical density measured at 600 nm, was centrifuged at 4000 rpm for 15 min. The bacterial pellet was twice washed in deionised water and then resuspended in deionised water. The initial cell density was 10^8 CFU mL^{-1}. The *E. coli* concentration was determined by viable cell count plate.

4.2.4 Fluorescence Microscopy

Appropriate amounts of silver flakes (RF and CF) were added in 50 mL of *E. coli* culture in deionised water medium. 1 mL of the samples was collected at initial and final time. Then the samples were treated with the DNA double staining Sybr Green I, SG (Molecular Probes, Eugene, Oregon, USA) and Propidium Iodide, PI (Sigma, St. Louis, Montana, USA) and observed at a fluorescence microscope. The sampling and analysis was done in triplicate.
Furthermore the samples were plated on an LB agar plate and incubated at 37 °C for 24 h. The colonies were counted by viable cell counts.
We subjected to this procedure also control samples.

4.2.5 Scanning Electron Microscopy (SEM)

0.5 g of reduced flakes (RF) were added in 50 mL of *E. coli* culture in deionised water medium. At 0 and 180 minutes 100 μL of the samples were plated on an LB agar plate and incubated at 37 °C for 24 h.
Then the samples were:

- fixed for 30 minutes with glutaraldehyde (2 %),

paraformaldehyde (1 %), picric acid (1 %) and $HgCl_2$ (0.1 %) in cacodylate buffer (0.1 M);
- washed three times for 10 minutes in cacodylate buffer solution (0.2 M);
- post-fixed with osmium tetroxide (1 %) in cacodylate buffer for 45 minutes;
- washed three times for 10 minutes with cacodylate buffer solution (0.2 M);
- drained with ethanol/water in increasing concentrations of ethanol (20 %, 50 %, 70 % x 2, 90 % and 100 % x 2) for 10 minutes. The absolute ethanol was replaced by dimethoxymethane;
- underwent critical point drying with CO_2;
- were located on a stub and metallized with gold.

The samples were microscoped and photographed with a scanning electron microscope (SEM LEO 1240). Analyses were carried out at CIMA, Milano University.
We subjected to this procedure also a control sample.

4.3 Results and discussion

With the aim to study the mechanism of action of silver flakes on bacterial cells, samples were collected from *E. coli* cultures added with activated flakes and treated with Sybr Green I, SG, and Propidium Iodide, PI. SG and PI make specific bonds with microbial DNA, but their ability to penetrate the cell through plasmatic membranes is different: SG can penetrate both viable and dead cells, while PI penetration concerns only membrane compromised cells, which can be simply damaged or definitively dead. So, in viable cells only SG is present, while in damaged or dead cells both

fluorochromes are found. When both fluorochromes are present at the same time, the light energy emitted by SG is used, by energy transfer, to excite PI. Viable cells thus emit only green fluorescence, dead cells only red fluorescence and damaged cells both green and red, according to the amount of PI which can penetrate the cell, depending on the damage extent.
4 g of RF were added to 50 mL of *E. coli* culture in deionised water. Samples were collected at 0, 180 and 1440 minutes. In all experiments the *E. coli* concentration was constant over time in control samples. The *E. coli* concentration, when exposed to RF, was zero at 180 and 1440 minutes.
1 mL samples were stained with Sybr Green (SG) and Propidium Iodide (PI). The results of fluorescence microscopy observations were reported in figure 1, as the mean of three replicates.

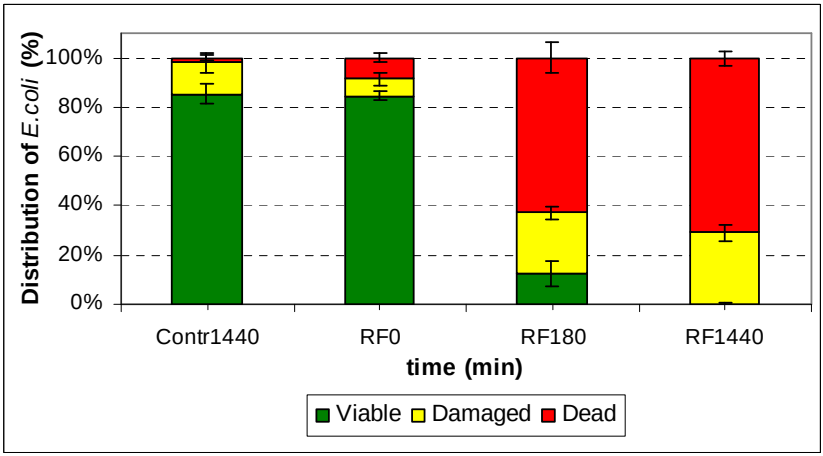

Figure 1 – *RF Fluorescence microscopy results.*
Mean values are reported with standard deviation.

In control samples, the viable cells percentage was constant over time (about 85 %) and the damaged and dead cells were < 10 %. The

same result was obtained for the *E. coli* treated with RF at 0 minutes. *E. coli* treated with RF at 180 minutes were damaged (25 %) and dead (63 %). At 1440 minutes damaged and dead *E. coli* were 29 % and 71 %, respectively. Furthermore, at both experimental times, the ratio dead (%) : damaged (%) was constant and equal to 2.5. On the basis of these results, we concluded that RF not only damaged the cells but also caused their death.

1 g of CF were added to 50 mL of *E. coli* culture in deionised water. Samples were collected at 0 and 6 minutes. In all experiments the *E. coli* concentration was constant over time in control samples. The *E. coli* concentration, when exposed to CF, was zero after 6 minutes. 1 mL samples were stained with Sybr Green (SG) and Propidium Iodide (PI). The results of fluorescence microscopy observations were reported in figure 2, as the mean of three replicates.

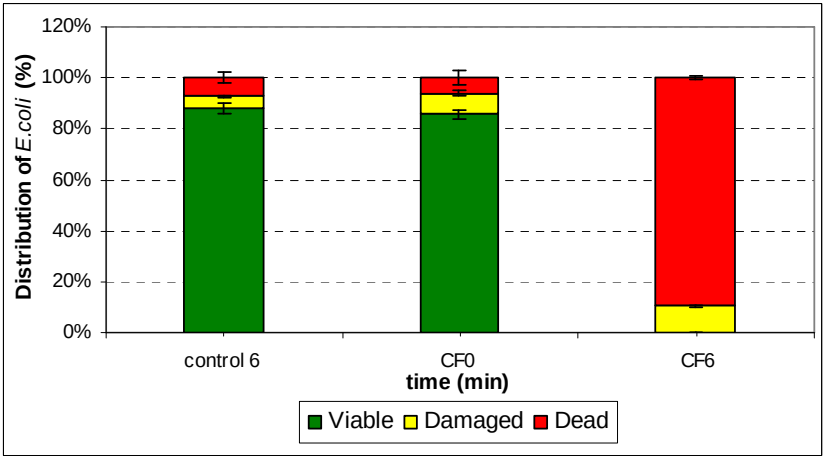

Figure 2 - *CF Fluorescence microscopy results.*
Mean values are reported with standard deviation.

In control samples, the viable cells percentage was constant over time (about 86-90 %) and the damaged and dead cells were < 10 %. The same result was obtained for the *E. coli* treated with CF at 0 minutes. After 6 minutes damaged and dead *E. coli* were 10 % and 90 %, respectively, the ratio dead (%) : damaged (%) being 8.7, higher than in the experiments with RF. So we concluded that CF principally caused the cell death in a shorter time.

Moreover we investigated the flakes effect on the *E. coli* cells using scanning electron microscopy technique. We underline that the *E. coli* concentration in sample control was constant at 0 and 180 minutes. While the concentration of *E. coli* treated with RF was zero at 180 min.

In figure 3 the SEM photographs of *E. coli* sample before (a) and after (b) treatment with RF were reported. In figure 3(a) the *E. coli* cells morphology is regular and don't appear damaged. While in figure 3(b) the *E. coli* cells can't be distinguished. The "objects" in figure 3(b) have various dimensions which in some cases are bigger than the normal dimension of *E. coli*. We supposed that they were the aggregates of the cells residues or the collapsed cells. So the RF treatment caused the cells damage and death.

We concluded that RF could be classified as bactericidal agent.

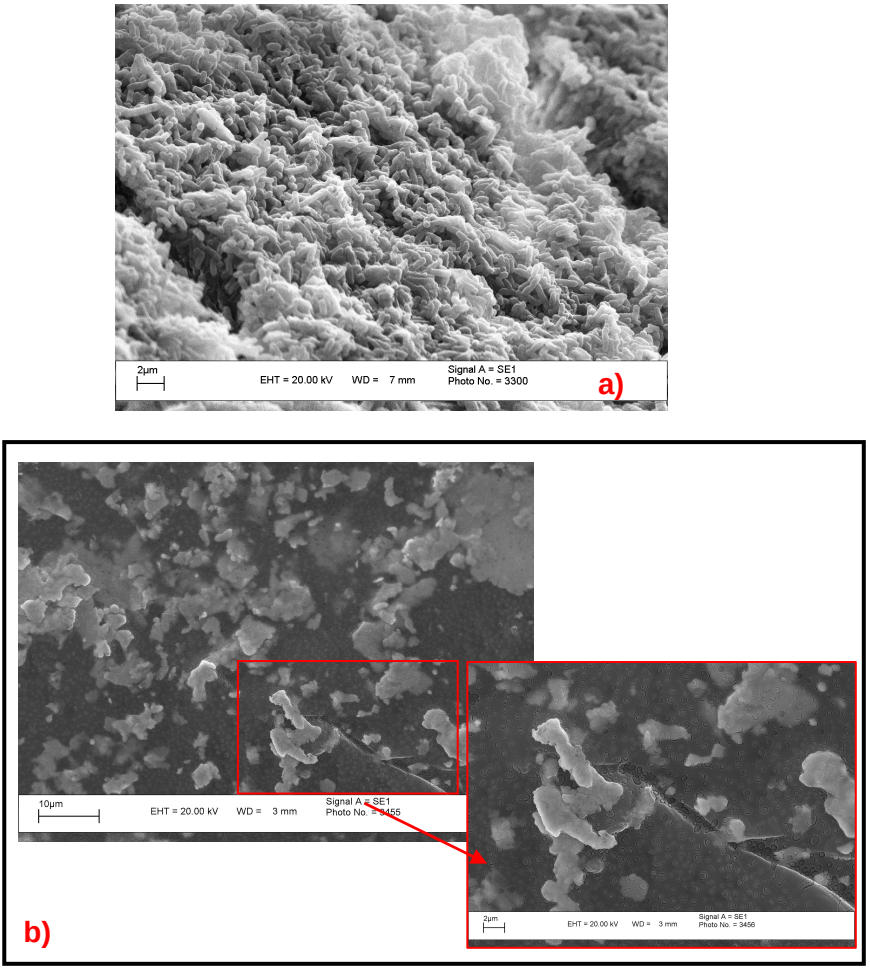

Figure 3 – *SEM photographs of E. coli before (a) and after (b) treatment (180 min) with RF.*

4.4 Conclusions

The recovery of a waste product from a thin metallic silver films manufacturing company was proposed using it as antimicrobial agent. The silver flakes were treated with thermal activation in reducing atmosphere and chemical activation. The flakes obtained by the thermal activation process in reducing atmosphere were named reduced flakes (RF) and the flakes obtained by the chemical activation process were named chemical flakes (CF). *E. coli* was used as indicator organism. The activated flakes (RF and CF) acquired antimicrobial properties.

The *E. coli* cells treated with activated flakes in aqueous medium were observed with fluorescence microscopy and scanning electron microscopy (SEM). We determined that the cells were damaged and at the end dead.

We concluded that activated flakes could be classified as bactericidal agent.

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Chapter 5

Release of Silver in Water

Abstract

In this work we studied the capability of thin silver flakes to release silver in aqueous medium by inductively coupled plasma optical emission spectrometry (ICP-OES). The flakes are the waste product of an Italian metallurgic company and they were previously treated with three different activation processes: thermal activation in reducing atmosphere, thermal activation in air and chemical activation. The Ag concentration released by flakes treated with either of the activation processes increased with contact time, instead the silver eluted from untreated flakes remained constant. We also verified that a relationship existed between the flakes concentration and the silver release. Furthermore, using *Escherichia coli* as Gram-negative microorganism model, we investigated if the antimicrobial property of the activated flakes was due only to the silver release. The presence of flakes was necessary to have *E. coli* decrease over time.

5.1 Introduction

Silver-based antimicrobials capture much attention not only because of the non-toxicity of the active Ag^+ to human cells [1, 2], but because of their novelty being a long lasting biocide. Silver ions are significant antimicrobials by virtue of their antiseptic properties [3, 4] with only few bacteria being intrinsically resistant to this metal [5]

The antimicrobial activity of silver is usually dependent on the silver cations Ag^+, which bind strongly to electron donor groups in biological molecules containing sulphur, oxygen or nitrogen. Hence the silver-based antimicrobial materials have to release the Ag^+ to a pathogenic environment in order to be effective. The oxidation of the

metallic silver to the active species Ag^+ was proposed to take place through an interaction of the silver with the water molecules [6].
For silver filled polymers, the entire process of silver ion release from the material was interpreted as the sum of three elementary processes. The diffusion of water into the composite specimen, the reaction between the silver and water molecules leading to the formation of silver ions and the migration of silver ions through the composite specimen leading to the release from the composite specimen to the aqueous environment. Having slower rates one of these physical processes can be the rate determining stage of the entire release mechanism. So material properties like the crystallinity and matrix polarity, which constitute the diffusion barrier to water molecules and the Ag^+ ions during their propagation through the specimens, can influence the rate of release [7]. In an effort to understand the mechanism of antimicrobial action of thin silver flakes, in this work we studied the capability of thin silver flakes to release silver in aqueous medium by inductively coupled plasma optical emission spectrometry (ICP-OES), as a function of contact time and varying the silver flakes concentration.

5.2 Methods and materials

5.2.1 Flakes features

An Italian company produced thin silver films starting from silver laminates, with a thickness of 10 µm, and using cold beating processes with mechanical hammers. During the cold beating process silver films, with different thickness, were produced. The films obtained in the final cold beating stage were cut, giving a square shape (10 x 10 cm), and inserted in a box. The residues, produced during this step, in the following named untreated flakes (UF), were used in this work.

The UF showed a sub-micrometric dimension: thickness was 300 nm. The UF thickness was measured by a contact stylus profilometer (KLA-TENCOR P10). Because silver flakes reacted electrostatically with charge surface we used a physical vapour deposition silver thin film as holder. In this way the properties of the substrate and the flakes had a good matching. The height and lateral resolution were, respectively, 2 nm and 0.8 µm [8].

The flakes didn't show homogeneous dimension. We determined the dimensional composition of flakes in percentage (table 1) using sieves with different porosity.

Table 1 - *Dimensional distribution (%) of untreated flakes (UF).*

≥2 mm	850 µm < d < 2 mm	500 µm < d < 850 µm	250 µm < d < 500 µm	150 µm < d < 250 µm	<150 µm
56 %	**23 %**	**10 %**	**7 %**	**3 %**	**1 %**

Furthermore organic impurities present in the flakes were determined by CHN elemental analysis: 0.13 % C, 0.02 % H, 0.01 % N.

5.2.2 Flakes activation processes

The untreated flakes were treated by three different activation processes:

1) Thermal activation in reducing atmosphere (TRA);
2) Thermal activation in air (TA);
3) Chemical activation (CA).

In **TRA** process, UF were heated at 710 °C for 60 seconds in an industrial furnace characterised by reducing atmosphere, which was formed by the ionic dissociation of ammonia (NH_3) at 890 °C. Then the flakes were cooled in the furnace for 3 minutes. The flakes obtained with this process were named reduced flakes (**RF**).

TA process used the same thermal conditions as the TRA process, but it took place in air. The obtained flakes were named thermal flakes (**TF**).

In **CA** process, UF were rinsed, under agitation, in hydrogen peroxide (H_2O_2) 35 % wt. (Sigma-Aldrich).
The reaction of silver with H_2O_2 is exothermic, producing warm and water vapour. When the reaction stopped the flakes were washed with sterilised deionised water and then were dried in an oven at 105 °C. The flakes obtained were named chemical flakes (**CF**).

5.2.3 Quantitative analysis of dissolved silver in water

We conducted two different experiments:

1) 0.01 g of flakes (UF, RF, TF and CF) were soaked, in steady condition, in 20 mL of deionised water for different contact times between 5 and 240 min and at selected times 10 mL of the eluate were sampled and analysed;
2) Different amounts of RF (0.01 g, 0.05 g and 0.1 g) were immerged, in steady condition, in 20 mL of deionised water for 40 min and then 10 mL of eluate were withdrawn.

The eluates were analysed with a Jobin-Yvon 38 (Horiba) inductively coupled plasma-optical emission spectrometry (ICP-OES). The sample was nebulised than transferred to an argon

plasma. It was decomposed, atomized and ionized whereby the atoms and ions were excited. We measured the intensity of the light when the atoms or ions returned to lower level of energy. Silver emitted energy at characteristic wavelength (328.07 nm) and it was used for quantitative analysis. The detection limit was 70 ppb. The experiments were repeated three times.
Note that ICP-OES didn't distinguish between forms of dissolved silver, so for the hereafter of this chapter we refered to dissolved silver (Ag^{dis}).

5.2.4 Microorganism and culture conditions

The bacterial strain used in this work was Gram-negative *Escherichia coli* (*E. coli* JM109). This strain came from our laboratory collection. *E. coli* is normally used as indicator organism in tests of environmental bacterial contamination. All materials used in the experiments were autoclaved at 121 °C for 30 min to ensure sterility. *E. coli* liquid cultures were prepared in 100 mL of LB medium (yeast extract 5 g/L, tryptone 10 g/L, NaCl 5 g/L) and incubated overnight at 37 °C by constant agitation. A known volume of the liquid culture, chosen on the basis of its initial optical density measured at 600 nm, was centrifuged at 4000 rpm for 15 min. The bacterial pellet was twice washed in deionised water and then resuspended in aqueous solutions containing silver (see. par. 5.2.5 and 5.2.6). The initial cell density was 10^8 CFU mL^{-1}.
The *E. coli* concentration were determined by the viable cell count plate.

5.2.5 Silver ions antimicrobial activity tests

The Ag^+ solutions of desired concentrations were prepared using $AgNO_3$ (99 %, Sigma-Aldrich) in deionised sterilised water.

With the aim to investigate the capability of silver ions to kill microorganisms (*E. coli*), the Ag^+ solutions were used as medium in antimicrobial tests. At fixed times, the samples were collected and serially diluted in the range of 10^{-1} to 10^{-8}. 100 µL of each dilution were plated on an LB agar plate. The colonies were counted after incubation at 37 °C for 24 h.
We also monitored the *E. coli* concentration in a control sample using only deionised sterilised water as aqueous medium.

5.2.6 Dissolved silver antimicrobial activity tests

4 g of reduced flakes (RF) were immerged in 50 mL of deionised sterilised water for 45 min under agitation at room temperature (22-23 °C). RF were separated from the medium using a membrane filter unit with a pore size of 1.2 µm (Whatman GC/F). To the recovered eluate (25 mL), deionised sterilised water was added to a final volume of 50 mL. This solution was used as aqueous medium for the antimicrobial tests.
At fixed times, the samples were collected and serially diluted in the range of 10^{-1} to 10^{-8}. 100 µL of each dilution were plated on an LB agar plate. The colonies were counted after incubation at 37 °C for 24 h.
We also monitored the *E. coli* concentration in a control sample using only deionised sterilised water as aqueous medium.

5.3 Results and discussion

The ICP-OES analysis of the eluate showed that each type of flakes (UF, RF and TF) released silver in deionised water (figure 1).

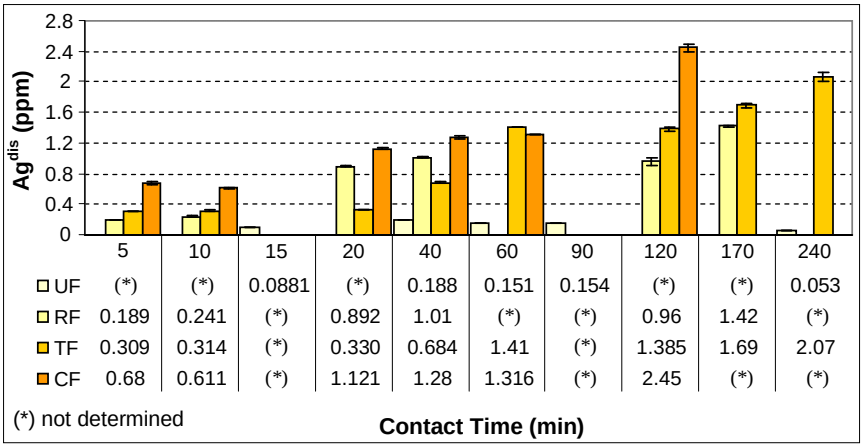

Figure 1 – *Ag^{dis} released in water.*

The trends observed for the Ag^{dis} concentration as a function of time were different for the four types of flakes.
The UF released about 0.1-0.2 ppm of silver until 90 min of contact time and at 240 min the concentration diminished.
The RF until 10 min of contact time eluted about 0.2 ppm of silver. This value was comparable with that measured for UF. With 20 min of contact time the silver reached the value of 0.9 ppm which was measured until 120 min. When the RF was immerged in water for 170 min, we detected about 1.4 ppm.
Silver released by TF until 20 min of contact time was constant, about 0.3 ppm. Then it increased gradually and reached a concentration of 2 ppm at 240 min of contact time.
CF released 0.6-0.7 ppm of silver until 10 min. Then the

concentration increased and reached a value of 2.5 ppm at 120 min of contact time.
In figure 2 the release of silver as a function of the contact time, until 170 min, was reported

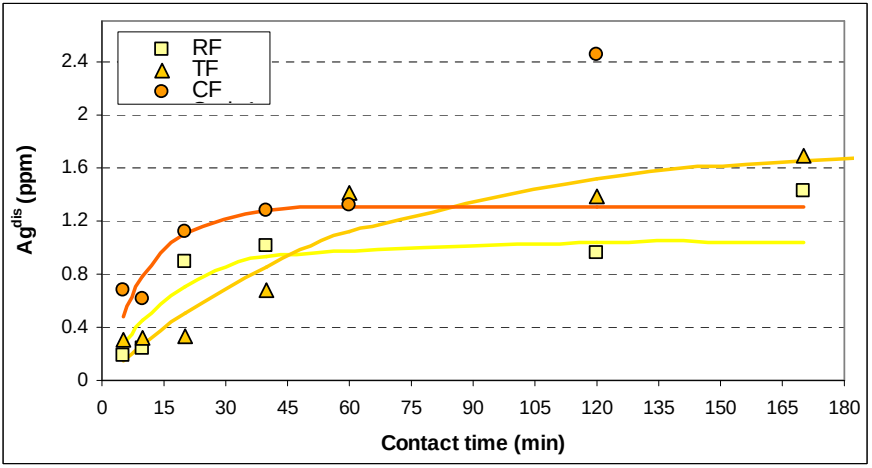

Figure 2 – *Release of silver from flakes (RF, TF and UF) as a function of contact time together with the calculate model.*

The Ag^{dis} released by RF, TF and CF increased with the contact time. At initial contact times, the Ag^{dis} released by CF was greater than RF, which in turn released higher amounts than TF.
At longer contact times, the Ag^{dis} seemed to reach a constant value.
The variation in Ag^{dis} concentration with time was modelled hypothesizing a first-order kinetics:

$$\mathbf{Ag^{dis} = Ag^{dis}_{\infty} \cdot (1 - \exp(-k\,t))}$$

where Ag^{dis}_{∞} was the concentration released at equilibrium and k (min^{-1}) the first-order kinetic constant of the release process. Both parameters were determined by a non-linear estimation procedure.

The calculated curves were reported in figure 2, showing a good agreement with the experimental data.
Estimated parameters were reported in table 2, together with the determination coefficient, R^2.

Table 2 - *Estimated parameters and R^2 values.*

	Ag_{∞}^{dis} (ppm)	*k* (min^{-1})	R^2
RF	**1.04**	**0.057**	**0.856**
TF	**1.75**	**0.017**	**0.910**
CF	**1.31**	**0.092**	**0.842**

The values of *k* reflected the slope of the initial part of the curve, *i.e.* the release rate was higher for CF; the Ag_{∞}^{dis} values reflected the asymptotic values approached by the curve at longer kinetic times.
Furthermore we showed that release of Ag^{dis} from RF in water was concentration dependent (figure 3).

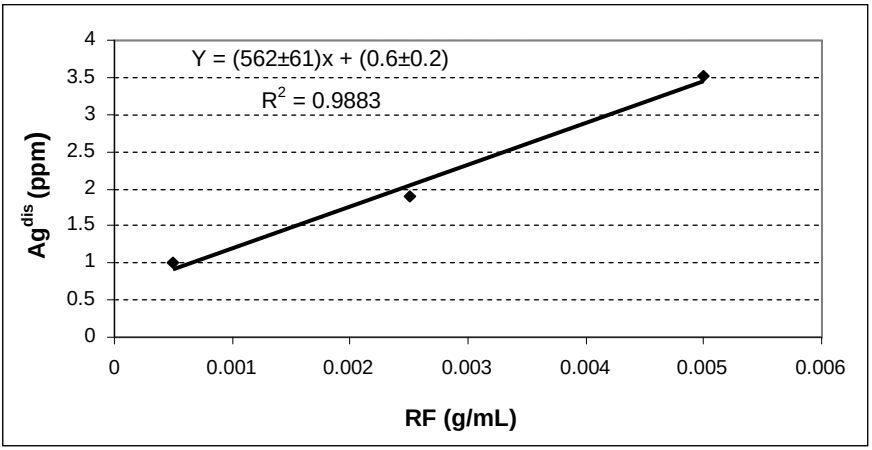

Figure 3 – *Dependence of release of Ag^{dis} with the RF concentration.*

It was evident that the concentration of Ag^{dis} was proportional to the RF concentration. In figure 3 the regression line that interpolated the experimental data was reported together with the line equation and R^2 value.

Kumar and Münstedt [6] studied the Ag^+ release from polyamide/silver composites and found that silver was oxidized in Ag^+ in aqueous medium. They observed that Ag^+ concentration in solution increased initially and then was followed by a marginal increase. As to own knowledge the release of silver from the flakes we are studying wasn't yet determined.

The trends observed for Ag^{dis} concentration in own experiments (figure 2) was similar to that in [6], we therefore hypothesised that the silver was released in water as Ag^+.

From the regression line equation in figure 3, we calculated the silver concentration in aqueous medium released by 0.001 g mL^{-1} of RF (1.2 ppm) and estimated the concentration released by 0.04 g mL^{-1} of RF (23 ppm).

$AgNO_3$ solutions of the two concentrations determined were used as medium for antimicrobial tests. In both tests, with a contact time lower than 1 min, we detected zero CFU mL^{-1}. *Escherichia coli* in presence of silver ions ($AgNO_3$) decreased much faster than in presence of 0.001 g mL^{-1} and 0.04 g mL^{-1} of RF (see par. 3.3.2).

On the basis of these results we supposed that the silver detected in water by ICP-OES wasn't silver ion.

Two further antimicrobial tests were conducted using as aqueous medium the water recovered by filtration from a 0.04 g mL^{-1} RF water suspension (see par. 5.2.6).

The results were shown in figure 4. The medium used didn't show any antimicrobial activity.

In absence of RF we didn't detect antimicrobial activity. This result was in disagreement with the antimicrobial tests realized using a solution of Ag^+.

We concluded that it was necessary the presence of flakes to have the *E. coli* diminished in aqueous solution.
We concluded that the presence of flakes was necessary to have the *E. coli* diminished in aqueous medium.

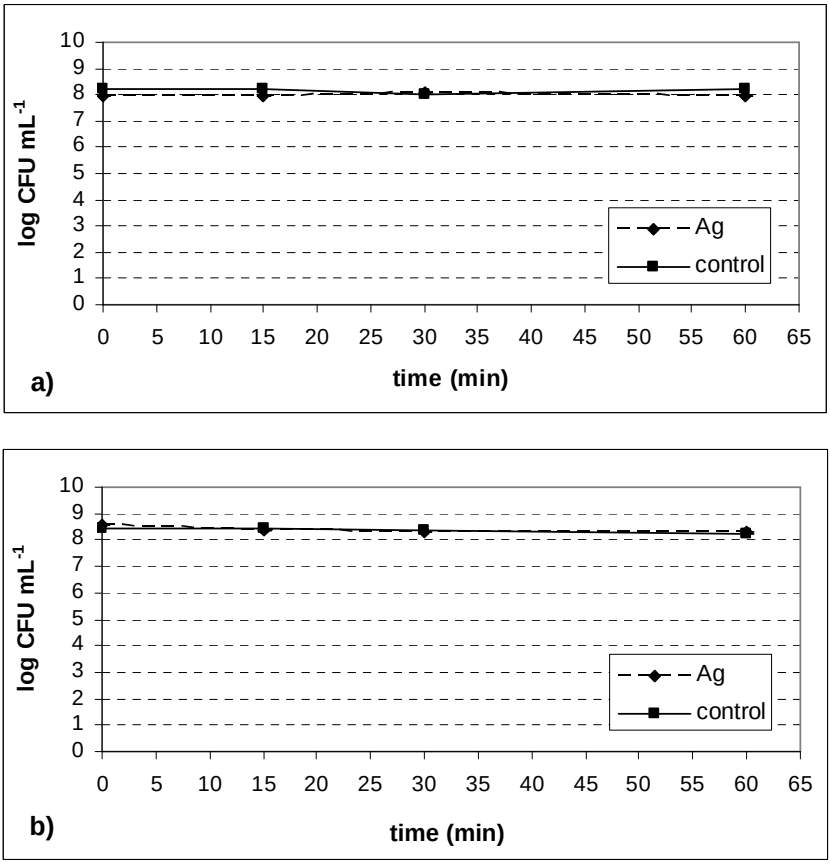

Figure 4(a,b) - *Dissolved silver antimicrobial activity tests.*

5.4 Conclusions

The recovery of a waste product from a thin metallic silver films

manufacturing company was proposed using it as antimicrobial agent. The silver flakes were treated with thermal activation in reducing atmosphere, thermal activation in air and chemical activation. The flakes obtained by the thermal activation process in reducing atmosphere were named reduced flakes (RF), the flakes obtained by the thermal activation process in air were named thermal flakes (TF) and the flakes obtained by the chemical activation process were named chemical flakes (CF). In chapter 3 we concluded that the activated flakes (RF, TF and CF) acquired antimicrobial property and so in this work we investigated if this capability was due to the silver released in water.

With ICP-OES analysis we determined that the silver released in water, by the activated flakes, increased with the contact time. At short contact times, silver released by CF was greater than RF, which in turn released higher amounts than TF. Furthermore we showed that release of Ag^{dis} from RF in water was concentration dependent.

We hypothesised that the silver dissolved in water was in ionic form. Eluates, obtained by filtration of RF immerged in water for 45 min, were used as medium for antimicrobial tests with *E .coli* as indicator organism. We didn't detect any antimicrobial activity; so we concluded that the presence of flakes was necessary to kill microorganisms.

$AgNO_3$ solutions of the two determined concentration (1.2 ppm and 23 ppm) were used as medium for antimicrobial tests. In both tests, with a contact time lower than 1 min, we detected zero CFU mL^{-1}. *Escherichia coli* in presence of silver ions ($AgNO_3$) decreased much faster than in presence of 0.001 g mL^{-1} and 0.04 g mL^{-1} of RF (see par. 3.3.2). Probably the silver dissolved detected with ICP-OES wasn't in ionic form.

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Chapter 6

Surface Characterisation of Thin Silver Flakes with X-ray Photoelectron Spectroscopy and Scanning Electron Microscopy

Abstract

In this work we investigated the surface of thin silver flakes. The flakes are the waste product of an Italian metallurgic company and they were previously treated with three different activation processes: thermal activation in reducing atmosphere, thermal activation in air and chemical activation.
The morphology and chemical composition of the flakes surface were characterised by scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS).
We observed flakes surface with SEM. We determined that the activation processes modified the flakes surface.
With XPS we determined the chemical composition of surface and bulk after 5 minutes of mild sputtering of the flakes. The species of interest (Ag, O and C) were present both on the surface and in the bulk. The atomic percentages of O and C after sputtering decreased with a corresponding Ag increase. The XPS data indicated the presence of silver in Ag^0 oxidation state, while the presence of AgO and Ag_2O could be excluded. The not activated flakes were the only exception. They presented, after sputtering, two Ag3d components (367.3 eV and 369.2 eV). The former component could be ascribed to AgO.

6.1 Introduction

Some reports have suggested that the antimicrobial activity of silver loaded materials is realized via the elution of silver ions into the system containing microorganisms, leading to cell death through cell penetration and binding at specific sites to DNA, RNA, respiratory enzymes and cellular protein [1].

At the beginning of last century, Gibbard [2] was the first to systematically investigate the antimicrobial properties of silver. He hypothesised that the bactericidal properties of silver might be due to ionized silver, probably from silver oxide. In his experiments silver metal was subjected to different treatments: (i) silver was melted in air and cooled in hydrogen, or melted and cooled in hydrogen and showed no evidence of any inhibitive action; (ii) silver metal was heated and cooled in air, or heated in hydrogen and cooled in air and showed inhibition of microbial growth.

Lok et al. [3] showed that the silver released in water by partially oxidized nano-Ag hadn't antimicrobial efficacy and furthermore demonstrated that the antibacterial activity of silver nanoparticles was dependent on chemisorbed Ag^{+}. In chapter 5 we observed that silver released in water by the flakes didn't reduce the *E. coli* concentration. Thus we concluded that the antimicrobial effect of the activated flakes was not associated with the elution of silver ions.

Djokic and Burrell [4] showed that only silver films containing silver oxide (Ag_2O) showed antimicrobial activities.

Silver adsorbs oxygen onto the surface as atomic oxygen. Because atomic oxygen fits into the octahedral holes of the silver, oxygen atoms accumulate within the bulk of silver [5]. Some Authors attributed the bactericidal activity of silver to catalytic oxidation involving reactive oxygen species [6-9]. Silver catalytic activity was due to chemisorption of atomic oxygen on the surface of silver combined with freedom of movement of oxygen throughout the crystal lattice of the silver [10]. On the basis of this knowledge and because we demonstrated (chapter 5) that the presence of activated flakes in water was necessary to have antimicrobial effect, we conducted morphological and chemical characterisation of silver flakes surface by scanning electron microscopy (SEM) and X-ray

photoelectron spectroscopy (XPS) to verify if the flakes antimicrobial activity could be associated with these aspects.

6.2 Methods and materials

6.2.1 Flakes features

An Italian company produced thin silver films starting from silver laminates, with a thickness of 10 µm, and using cold beating processes with mechanical hammers. During the cold beating process silver films, with different thickness, were produced. The films obtained in the final cold beating stage were cut, giving a square shape (10 x 10 cm), and inserted in a box. The residues, produced during this step, in the following named untreated flakes (UF), were used in this work.

The UF showed a sub-micrometric dimension: thickness was 300 nm. The UF thickness was measured by a contact stylus profilometer (KLA-TENCOR P10). Because silver flakes reacted electrostatically with charge surface we used a physical vapour deposition silver thin film as holder. In this way the properties of the substrate and the flakes had a good matching. The height and lateral resolution were, respectively, 2 nm and 0.8 µm [11].

The flakes didn't show homogeneous dimension. We determined the dimensional composition of flakes in percentage (table 1) using sieves with different porosity.

Table 1 - D*imensional distribution (%) of untreated flakes (UF).*

≥2 mm	850 µm < d < 2 mm	500 µm < d < 850 µm	250 µm < d < 500 µm	150 µm < d < 250 µm	<150 µm
56 %	**23 %**	**10 %**	**7 %**	**3 %**	**1 %**

Furthermore organic impurities present in the flakes were determined by CHN elemental analysis: 0.13 % C, 0.02 % H, 0.01 % N.

6.2.2 Flakes activation processes

The untreated flakes were treated by three different activation processes:

1) Thermal activation in reducing atmosphere (TRA);
2) Thermal activation in air (TA);
3) Chemical activation in air (CA).

In **TRA** process, UF were heated at 710 °C for 60 seconds in an industrial furnace characterised by reducing atmosphere, which was formed by the ionic dissociation of ammonia (NH_3) at 890 °C. Then the flakes were cooled in the furnace for 3 minutes. The flakes obtained with this process were named reduced flakes (**RF**).

TA process used the same thermal conditions as the TRA process, but it took place in air. The obtained flakes were named thermal flakes (**TF**).

In **CA** process, UF were rinsed, under agitation, in hydrogen

peroxide (H_2O_2) 35 % wt. (Sigma-Aldrich).
The reaction of silver with H_2O_2 is exothermic, producing warm and water vapour. When the reaction stopped the flakes were washed with sterilised deionised water and then were dried in an oven at 105 °C. The flakes obtained were named chemical flakes (**CF**).

6.2.3 Scanning electron microscopy (SEM)

The morphologies of the flakes (UF, RF, TF and CF) were observed on a scanning electron microscopy SEM (TESCAN VEGA TS 5136 XM, analyses were carried out at the department of Material Science, Milano-Bicocca University).
The samples were mounted on carbon stubs.

6.2.4 X-ray photoelectron spectroscopy (XPS)

The chemical composition of silver flakes (UF, RF, CF and r-CF) on the surface and after 5 min of a mild sputtering was investigated by XPS. r-CF sample corresponded to the CF sample when reused (see par. 3.3.1.3).
Analyses were carried out by ISTM-CNR, Padova University.

XPS spectra were collected using a Perkin-Elmer Φ5600ci spectrometer using standard Al-K_α radiation (1486.6 eV) operating at 350 W. The working pressure was < $5 \cdot 10^{-8}$. The calibration was based on the binding energy (BE) of the values $Au4f_{7/2}$ line at 83.9 eV with respect to tha Fermi level. The standard deviation for the BE values was 0.15 eV. The reported BE's were corrected for the BE's charging effects assigning the BE value of 284.6 eV to the C1s line of carbon [12] in the outer layers where contamination carbon is still present. Survey scans were obtained in the 0-1300 eV range

(pass energy 187.85 eV, 1.0 eV/step, 50 ms/step). Detailed scans (58.7 eV pass energy, 0.1 eV/step, 100-150 ms/step) were recorded for the O 1s, C 1s, Ag 3d, Ag 3p, Ag MVV, Sn 3d and N 1s regions. The atomic composition, after a Shirley type background subtraction [13], was evaluated using sensitivity factors supplied by Perkin-Elmer [14]. Peak assignment was carried out according to literature data [12, 14, 15].
Depth profiles were carried out by Ar^+ sputtering at 3 kV with an Argon partial pressure of $5 \cdot 10^{-6}$ Pa. A specimen area of 2x2 mm^2 was sputtered. Samples were introduced directly, by a fast entry lock system, into the XPS analytical chamber.

For a detailed analysis, the core-level lines obtained by XPS were fitted by the freeware program XPS Peak 41 by subtracting a Shirley background and by using Gaussian-Lorentzian contributions. Elemental analyses were carried out by using the home-made program XPS HITS Version 4.7 (2008), by using the corresponding photoionisation cross-sections [14].

6.3 Results and discussion

6.3.1 Scanning electron microscopy

The SEM images obtained for the different flakes (UF, RF, TF and CF) showed some changes in the morphology of the flakes surface after the activation processes (figure 1).
The image of figure 1(a) was taken from the Untreated Flakes (UF). The scratches caused by the manufacturing process were clearly visible on the sample surface. The images of figure 1(b) and figure 1(c) showed the surface of RF and TF, respectively. Apparently the two thermal activation processes, although conducted in two

different atmospheric conditions (reducing atmosphere and air) modified the flakes surface in the same way. Both RF and TF surfaces presented grains. The RF grains seemed smaller than TF grains. The image of figure 1(d) was taken from the CF sample. In this case we noted that the surface was homogeneous and similar to the UF sample, although the manufacturing scratches disappeared.

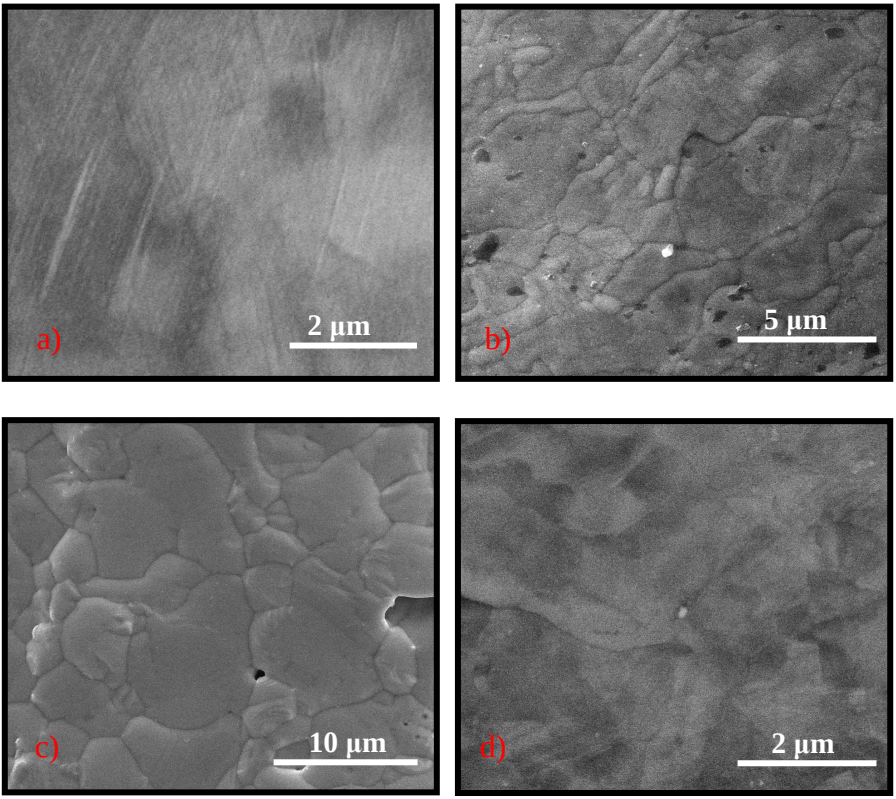

Figure 1 – *SEM images of (a) untreated flakes (UF) surface; and activated flakes surfaces: RF (b), TF (c) and CF (d).*

We concluded that only RF and TF surfaces showed a noticeable morphology change. We detected the formation of grains, probably due to the recrystallization process which was caused by the high temperature of the thermal treatment [29].

6.3.2 X-ray photoelectron spectroscopy

XPS was used to examine the chemical state changes which occurred on the surface of the silver flakes after activation processes.
For both untreated (UF) and activated flakes (RF, CF and r-CF) the elements identified were Ag, O and C. In CF and r-CF samples we found also traces (about 1%) of Sn and N; after mild sputtering these elements disappeared (figure 2).

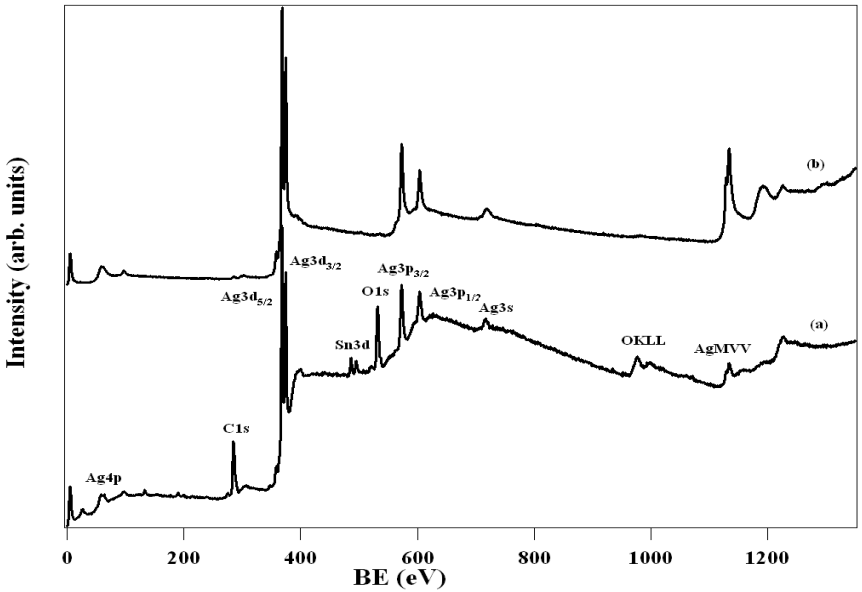

Figure 2- *Survey spectra of sample CF before (a) and after (b) sputtering.*

The atomic percentages obtained for the flakes (UF, RF, CF and r-CF) before and after mild sputtering were reported in table 2.

Table 2- Atomic percentages delivered by XPS analysis.

Sample	Atomic concentration (%)			O/Ag
	Ag	O	C	
UF	30.4	10.0	59.6	0.3
UF-sputtered	67.3	4.9	27.7	
RF	47.8	18.5	33.7	0.4
RF-sputtered	75.0	3.5	21.5	
CF	16.8	30.4	53.4	1.8
CF-sputtered	64.6	7.5	27.9	
r-CF	20.8	30.9	47.5	1.5
r-CF-sputtered	65.5	6.1	28.0	

We observed that oxygen and carbon percentages after mild sputtering decreased for all samples, while silver respectively increased. The bulk chemical composition could be considered similar for all the flakes except for RF sample where Ag% was greater and O% and C% were lower. So we concluded that the activation processes didn't change the bulk composition.
Regarding the surface, we observed that oxygen was present in different amounts. In the different samples the O/Ag atomic ratio was CF>r-CF>RF>UF. CF had a much higher amount of oxygen than RF and UF. This trend could be associated with the activation processes, in fact CF was treated with hydrogen peroxide. Oxygen on r-CF surface slightly diminished. In RF and UF samples the oxygen was lower.
The O/Ag trend for the flakes was similar to the trend observed for antimicrobial activity (see chapter 3). We therefore hypothesised that oxygen had a role in the antimicrobial activity.

The binding energies (BE) obtained by XPS for the different species before and after sputtering were reported in table 3.

Table 3 – *BE values of the O 1s, Ag $3d_{5/2}$ and Ag M_4VV lines together with the experimentally determined Auger parameters (values corrected for charging affects).*

Sample	BE O 1s (eV)	BE Ag $3d_{5/2}$ (eV)	KE Ag M_4VV (eV)	Auger parameter energy (eV)
UF	**530.6**	**368.3**	**358.0**	**726.3**
UF-sputtered	**530.9**	**367.3**	**358.0**	**725.3**
RF	**531.1**	**368.2**	**357.9**	**726.1**
RF-sputtered	**nd**	**368.0**	**358.1**	**726.1**
CF	**531.5**	**367.8**	**358.4**	**726.2**
CF-sputtered	**531.7**	**368.1**	**358.5**	**726.6**
r-CF	**531.2**	**368.0**	**358.2**	**726.2**
r-CF-sputtered	**531.2**	**368.1**	**358.2**	**726.3**

The flakes were characterized by Ag $3d_{5/2}$ spectra with binding energies between 367.8-368.3 eV. These BE values were in agreement with literature data for Ag(0) (367.9-368.3 eV) [14, 16-22]. However, BE value of CF (367.8 eV) in some works [23, 24] was associated to the presence of Ag(I) which was attributed to the formation of Ag_2O. Moreover for UF-sputtered we identified a lower BE value (367.3 eV) which was closer to the Ag^{2+} associated with the possible presence of AgO [24, 25] (figure 3). The higher peak (369.2 eV) was more difficult to be assigned.

Since the BE values of Ag $3d_{5/2}$ for different silver species were quite similar (Ag(0): 367.9-368.3 eV; Ag_2O: 367.5-367.9 eV; AgO: 367.1-367.9 eV; Ag_2CO_3: 367.3-367.8 eV) the evaluation of Auger parameter was important (Ag(0): 726-726.4 eV; Ag_2O: 724.3-724.5 eV; AgO: 724.0-725.1 eV; Ag_2CO_3: 723.1 eV)

[12, 15, 18]. Observing the experimental BE values and the corresponding Auger parameters (table 3), we concluded that Ag(0) was present in all samples and we ruled out the presence of silver oxide, with the exception of UF-sputtered.

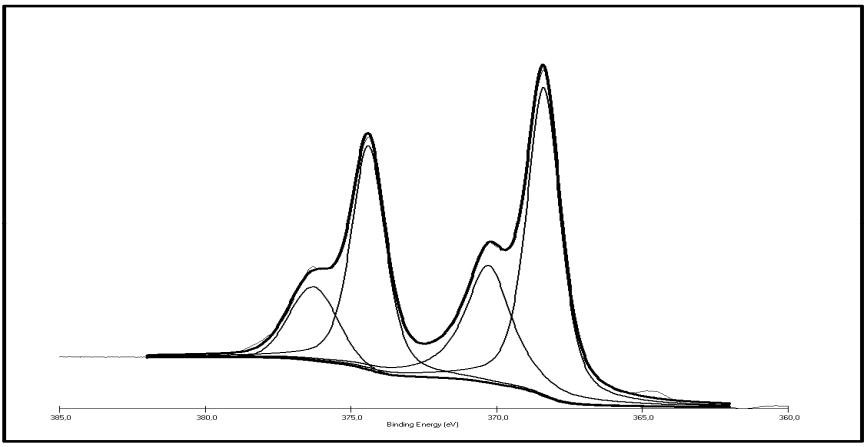

Figure 3- *Deconvoluted spectrum of Ag 3d region in UF sample after 5 minutes of sputtering.*

The C 1s spectrum, for all samples, contained a weak peak at about 287.5-288.0 eV which was in reasonable accord with literature data for carbon in carbonate on Ag surface (287.7-288.4 eV) [26].

The O 1s spectra, for all samples, showed a broad asymmetric peak at 531 eV (figure 4) containing contributions from several different oxygen-containing species. A major contributor to the peak envelope was a surface carbonate species, which exhibited an O 1s binding energy around 530.8 eV. Others possible contributors to the high binding energy tail of the O 1s peak were chemisorbed and dissolved

oxygen species (530.5-531.5 eV), hydroxyl species (531.7 eV) and chemisorbed H_2O (533.0 eV).

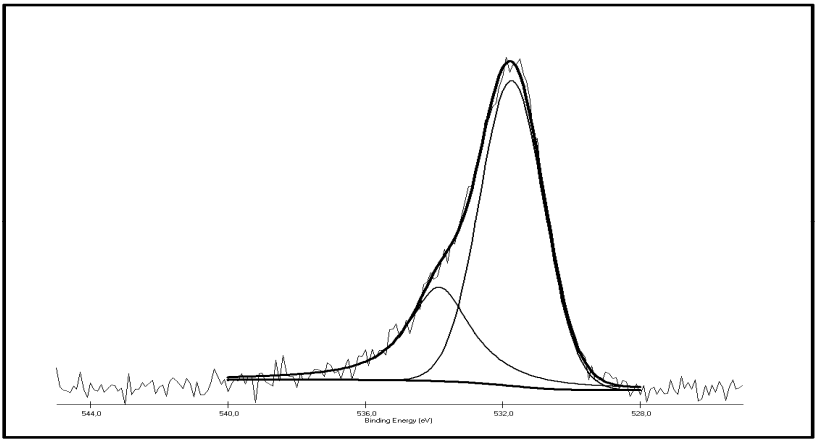

Figure 4 – *O 1s XPS data taken from RF sample*.

For the activated flakes (RF, CF and r-CF) the O 1s binding energy values were in agreement with O 1s data reported for bulk dissolved oxygen (530.5-531.5 eV) [27, 28]. For untreated flakes (UF) the O 1s binding energy was more similar to the values for carbonate on Ag surface (530.5-531.0 eV) [27]. However, no component ascribed to neither silver(I) oxide (528.8-529.0 eV) [14, 16-22] nor to silver(II) oxide (528.3-528.5 eV) [14, 16, 18-20] could be evidenced.

On activated flakes surface (RF, CF and r-CF), considering the basis of Ag $3d_{5/2}$ BE and Auger parameter values, we found Ag(0) and we didn't find Ag_2O or Ag^+. On untreated flakes we didn't rule out the presence of AgO.

Concerning the presence of oxygen species, these were more abundant in the activated samples. The main contributions deriving from surface carbonate species and dissolved oxygen species.
We concluded that the antimicrobial property acquired by activated flakes wasn't dependent on Ag_2O and Ag^+ chemical species [3, 4] but probably it was due to the presence of dissolved oxygen which was in higher amount on CF and r-CF samples.

6.4 Conclusions

In this work we characterised the flakes surface by Scanning Electron Microscopy (SEM) and by X-ray Photoelectron Spectroscopy (XPS).
We investigated the flakes surface morphology changes when treated with activation processes. We observed that after the two thermal activation processes (in reducing atmosphere and in air) on the flakes surface some grains with various dimensions appeared.
On the basis of XPS analysis we concluded that the antimicrobial property acquired by activated flakes wasn't dependent with Ag_2O and Ag^+ chemical species but probably it was due to the presence of dissolved oxygen which was in higher amount on CF and r-CF samples.

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Chapter 7

Applications

Abstract

In this work we investigated the use of thin metallic silver flakes as antimicrobial agent in aqueous phase. Flakes are waste products of a company producing silver films and so they are a cheaper way of using silver in applications when it is necessary to hinder the proliferation of microorganisms. Flakes acquired antimicrobial property by activation processes: thermal activation in reducing atmosphere and chemical activation.
We conducted two types of applicative experiments. We tested the antimicrobial capability of activated flakes (1) using tap water as medium and (2) on the effluent of a wastewater treatment plant.
We demonstrated that in both experiments the cell density was reduced.

7.1 Introduction

Pollution crisis is a major problem all around the world. It had adversely affected the lives of millions of people and caused many deaths and health disorder. Water pollution is one of the main causes for this crisis and the majority of water-borne diseases are spreading because of the poor quality of water, particularly due to the presence of bacteria and viruses in the water. Hence it is important to purify the water before its use [1]. There are several methods of water purification like chlorination [2], iodination [3, 4], ozonation [5], UV-purification [2], reverse osmosis [6]. However there are some disadvantages associated with these methods. For example the byproducts formed during chlorination are known as carcinogenic [7]. Recently many environmentally friendly inorganic bactericidal materials have been studied for disinfection in water.
On the basis of the results obtained in the previous chapters we tested the use of the activated silver flakes in applications when it is necessary to hinder the proliferation of microorganisms in aqueous phase. These flakes can easily be applied even without any

supporting medium, for example to the materials to be treated, or inserted in filters, devices or formulations.
At first we investigated the influence of the aqueous medium on the antibacterial activity of the flakes using tap water to resuspend *E. coli* culture. Thereafter, the activated flakes were used to treat the effluent of a wastewater treatment plant.

7.2 Methods and materials

7.2.1 Flakes features

An Italian company produced thin silver films starting from silver laminates, with a thickness of 10 µm, and using cold beating processes with mechanical hammers. During the cold beating process silver films, with different thickness, were produced. The films obtained in the final cold beating stage were cut, giving a square shape (10 x 10 cm), and inserted in a box. The residues, produced during this step, in the following named untreated flakes (UF), were used in this work.

The UF showed a sub-micrometric dimension: thickness was 300 nm. The UF thickness was measured by a contact stylus profilometer (KLA-TENCOR P10). Because silver flakes reacted electrostatically with charge surface we used a physical vapour deposition silver thin film as holder. In this way the properties of the substrate and the flakes had a good matching. The height and lateral resolution were, respectively, 2 nm and 0.8 µm [8].

The flakes didn't show homogeneous dimension. We determined the dimensional composition of flakes in percentage (table 1) using sieves with different porosity.

Table 1 - *Dimensional distribution (%) of untreated flakes (UF).*

≥2 mm	850 µm < d < 2 mm	500 µm < d < 850 µm	250 µm < d < 500 µm	150 µm < d < 250 µm	<150 µm
56 %	**23 %**	**10 %**	**7 %**	**3 %**	**1 %**

Furthermore organic impurities present in the flakes were determined by CHN elemental analysis: 0.13 % C, 0.02 % H, 0.01% N.

7.2.2 Flakes activation processes

The untreated flakes were treated by two different activation processes:

1) Thermal activation in reducing atmosphere (TRA);
2) Chemical activation in air (CA).

In **TRA** process, UF were heated at 710 °C for 60 seconds in an industrial furnace characterised by reducing atmosphere, which was formed by the ionic dissociation of ammonia (NH_3) at 890 °C. Then the flakes were cooled in the furnace for 3 minutes. The flakes obtained with this process were named reduced flakes (**RF**).

In **CA** process, UF were rinsed, under agitation, in hydrogen peroxide (H_2O_2) 35 % wt. (Sigma-Aldrich).
The reaction of silver with H_2O_2 is exothermic, producing warm and water vapour. When the reaction stopped the flakes were washed with sterilised deionised water and then were dried in an oven at 105 °C. The flakes obtained were named chemical flakes (**CF**).

7.2.3 Experiments with tap water as culture medium

7.2.3.1 Microorganism and culture conditions

The bacterial strain used in this work was Gram-negative *Escherichia coli* (*E. coli* JM109). This strain came from our laboratory collection. *E. coli* is normally used as indicator organism in tests of environmental bacterial contamination. All materials used in the experiments were autoclaved at 121 °C for 30 min to ensure sterility. *E. coli* liquid cultures were prepared in 100 mL of LB medium (yeast extract 5 g/L, tryptone 10 g/L, NaCl 5 g/L) and incubated overnight at 37 °C by constant agitation. A known volume of the liquid culture, chosen on the basis of its initial optical density measured at 600 nm, was centrifuged at 4000 rpm for 15 min. The bacterial pellets were twice washed and resuspended in tap water. The initial cell density was 10^7-10^8 CFU/mL.

7.2.3.2 Antimicrobial activity tests

4 g of reduced flakes (RF) were added in 50 mL of *E. coli* culture in tap water medium. The antibacterial activity was investigated in batch at room temperature (22-23 °C), monitoring the cell density by viable cell counts.
At fixed times, the samples were collected and serially diluted in the range of 10^{-1} to 10^{-8}. 100 µL of each dilution were plated on an LB agar plate. The colonies were counted after incubation at 37 °C for 24 h. The reproducibility of the plate counting method was within 0.4 log CFU/mL.
During every tests we also monitored the *E. coli* concentration in absence of flakes (control sample). In all the tests, the order of magnitude of the microbe count determined for the control remained unchanged over time.

7.2.4 Disinfection of the effluent of a wastewater treatment plant

1 g of RF or CF were added to 50 mL of the effluent. The antibacterial activity was investigated in batch at room temperature (22-23 °C), monitoring the cell density by viable cell counts.
At fixed times, the samples were collected and serially diluted in the range of 10^{-1} to 10^{-8}. 100 µL of each dilution were plated on an LB agar plate. The colonies were counted after incubation at 37 °C for 48 h. In these experiments we monitored the total heterotrophic bacteria. The initial cell density was 10^3 CFU/mL.

7.2.5 Statistical procedure

To verify whether the results of the experiments were significantly different, a statistical procedure for comparing slopes of regression lines was applied at 95 % confidence level [9, pp. 300-302]. The values were considered not different if the statistical test indicated that the null hypothesis H_0 (the values were not different at 95 % confidence level) was accepted; on the contrary, if H_0 was rejected, the values were different.
When the slopes were not significantly different we calculated the common slope (b_{com}) [9, pp. 292-295].

7.3 Results and discussion

7.3.1 Experiments with tap water as culture medium

To investigate the influence of the aqueous medium on the antibacterial activity of the activated flakes, we monitored the *E. coli* concentration over time in a culture suspended in tap water. In figure 1 the data points obtained in the experiments were reported.

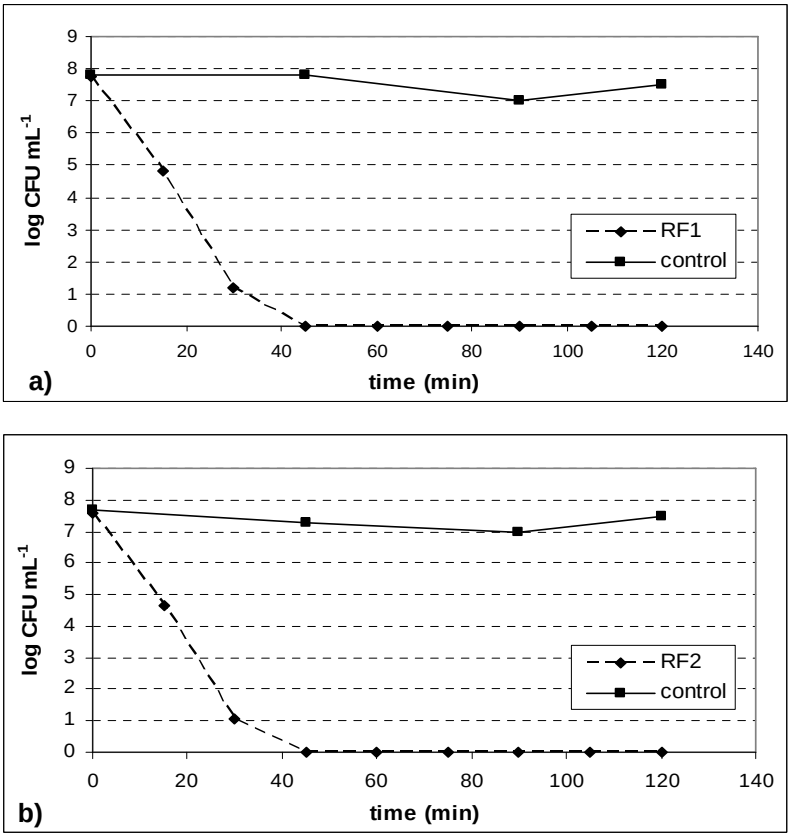

Figure 1(a-d) – *Antimicrobial activity of RF in tap water.*

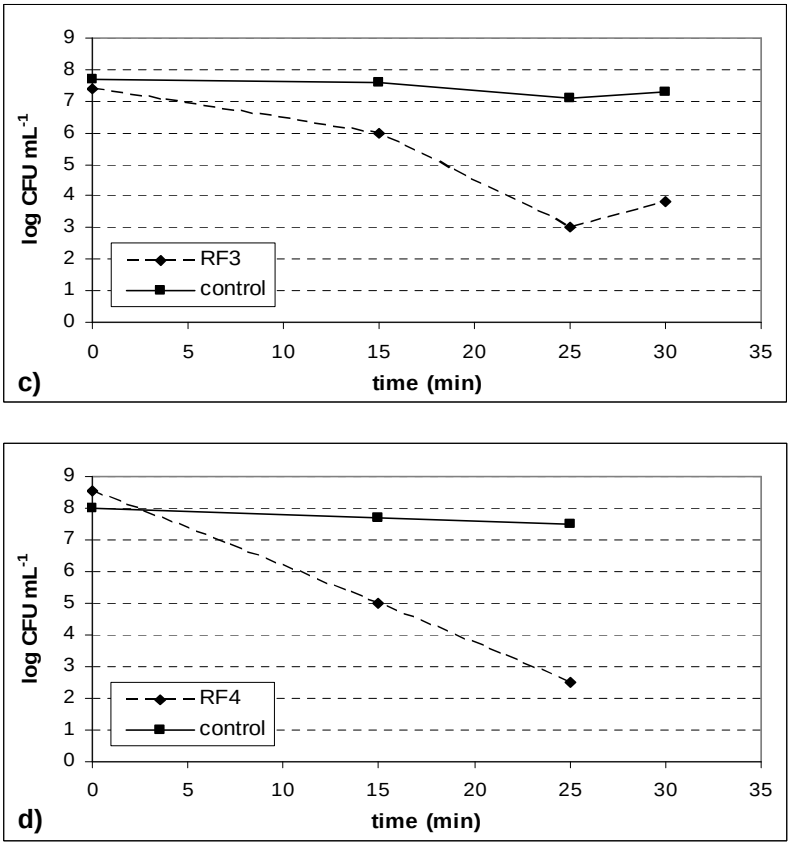

Figure 1(a-d) – *Antimicrobial activity of RF in tap water*.

The RF showed antimicrobial activity against *Escherichia coli* using tap water as medium. We calculated the regression lines and R^2 values for each experiment (table 2). The regression lines were calculated considering as final time the first data point with zero cell concentration.

Table 2 – *Regression line equations and correlation coefficients for each experiment.*

	Equation	R^2
RF *(2a)*	Y = (-0.18±0.02)x + (7.5±0.7)	0.9657
RF *(2b)*	Y = (-0.18±0.02)x + (7.3±0.7)	0.9623
RF *(2c)*	Y = (-0.14±0.04)x + (7.5±0.8)	0.8644
RF *(2d)*	Y = (-0.243±0.005)x + (8.57±0.08)	0.9996

The correlation coefficients (R^2) are higher than 0.9 except for RF (*2c*). On the basis of the regression lines and the R^2 values we concluded that the experimental data showed a linear trend. The same was observed for experiments performed using deionised water as culture medium (chapter 3).
The slopes of each regression line represented the decrease of viable cells, expressed as CFU/mL, in a minute and was termed kill rate [log CFU mL^{-1} min^{-1}]. The slopes of the regression lines determined in the experiments were compared applying the statistical test at 95 % confidence level [9, pp. 300-302] and we verified that the slopes were not significantly different. Then we calculated the common slope (b_{com} = 0.18±0.02 log CFU mL^{-1} min^{-1}) for the experiments [9, pp. 300-302].

We considered the common slope calculated in the experiments conducted with tap water (0.18±0.02 log CFU mL^{-1} min^{-1}) and the common slope calculated in the experiments conducted with deionised water (0.30±0.03 log CFU mL^{-1} min^{-1}) (see par. 3.3.2).
Using the common slopes, we estimated the time necessary to have zero CFU mL^{-1} using a different medium of suspension (tap water and deionised water) and the same amount of RF (4 g) and supposing to have an initial cell concentration of 10^8 CFU mL^{-1}. The estimated times were 44 min for real system (tap water) and 27 min for ideal system (deionised water).

We concluded that the kill rate in ideal conditions is greater than the kill rate in real conditions. Furthermore the RF antimicrobial activity is affected by the experimental medium. This result is in agreement with literature [10].

7.3.2 Disinfection of the effluent of a wastewater treatment plant

The feasibility of using silver flakes as an alternative disinfection agent for wastewater treatment plant effluent was investigated. We monitored the total heterotrophic bacteria concentration over time in presence and in absence (control) of activated silver flakes. In figure 2 and in figure 3 the data points for the experiments conducted using, respectively, RF and CF, were reported.

Both RF and CF showed antimicrobial activity on the effluent. As different microorganism species were present, we supposed that the activated flakes were active not only against the *E. coli* proliferation, but also against other bacteriological species.
The experimental data were elaborated as those obtained in the experiments in which *E. coli* concentration was monitored. Regression lines and R^2 values for each experiment were reported in table 3.

Table 3 – *Regression line equations and correlation coefficients for each experiment.*

	Equation	R^2
RF *(3a)*	Y = (-0.009±0.001)x + (3.0±0.2)	0.8606
RF *(3b)*	Y = (-0.012±0.002)x + (2.8±0.3)	0.8327
CF *(4a)*	Y = (-0.041±0.004)x + (3.20±0.06)	0.9621
CF *(4b)*	Y = (-0.031±0.002)x + (3.27±0.04)	0.9717
CF *(4c)*	Y = (-0.04±0.01)x + (3.1±0.2)	0.7573

The correlation coefficients (R^2) are higher than 0.8 except for CF (*4c*).
The slopes of each regression line represented the decrease of viable cells, expressed as CFU/mL, in a minute and was termed kill rate [log CFU mL^{-1} min^{-1}]. The slopes of the regression lines determined in the experiments were compared applying the statistical test at 95 % confidence level [9, pp. 300-302]. We verified that the slopes were not significantly different. Then we calculated the common slope for RF experiments (0.01 log CFU mL^{-1} min^{-1}) [9, pp. 292-295] and for CF experiments (0.037±0.005 log CFU mL^{-1} min^{-1}) [9, pp. 300-302].
It's evident that CF were more effective than RF. This result is in agreement with the results obtained against *E. coli* in deionised water.

We considered the common slopes calculated for the experiments conducted using RF on the effluent (0.01 log CFU mL^{-1} min^{-1}) and in deionised water (0.080±0.006 log CFU mL^{-1} min^{-1}) (see par. 3.3.2) and the common slopes calculated for the experiments conducted using CF on the effluent (0.037±0.005 log CFU mL^{-1} min^{-1}) and in deionised water (0.9±0.1 log CFU mL^{-1} min^{-1}) (see par. 3.3.3). Both RF and CF were more effective in ideal than in real conditions but it was evident that CF antimicrobial activity was more affected than RF by the experimental medium.
Using the common slopes, we estimated the time necessary to have zero CFU mL^{-1} (limit set by Italian standards for water reuse) in the effluent. The estimated times were 300 min using RF and 81 min using CF.

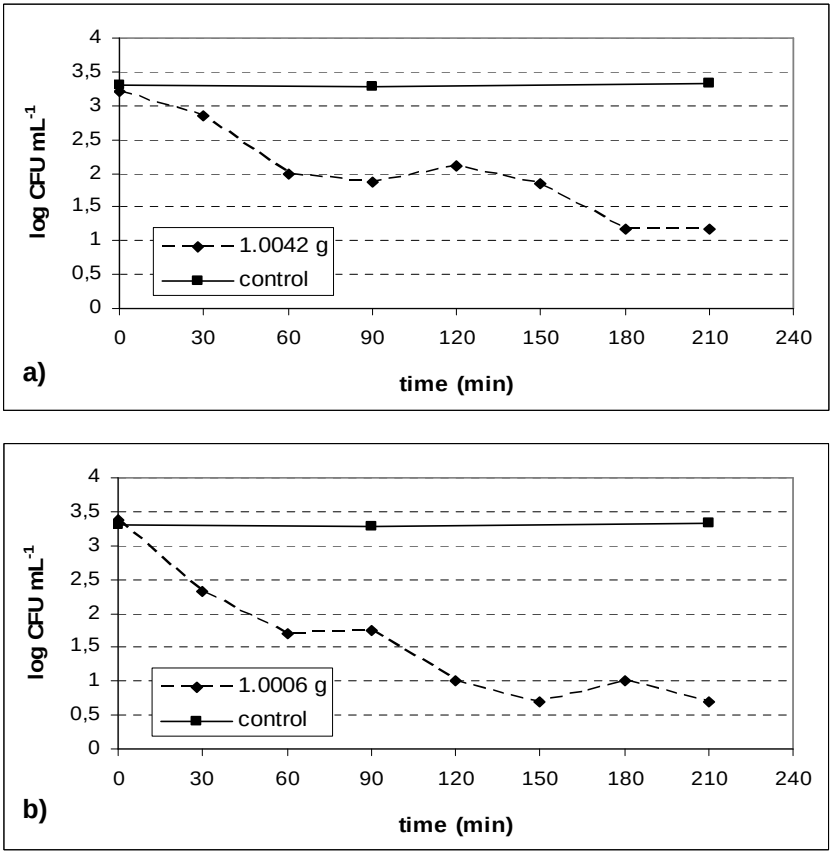

Figure 2 (a,b) – *Antimicrobial activity of RF on the effluent of a wastewater plant.*

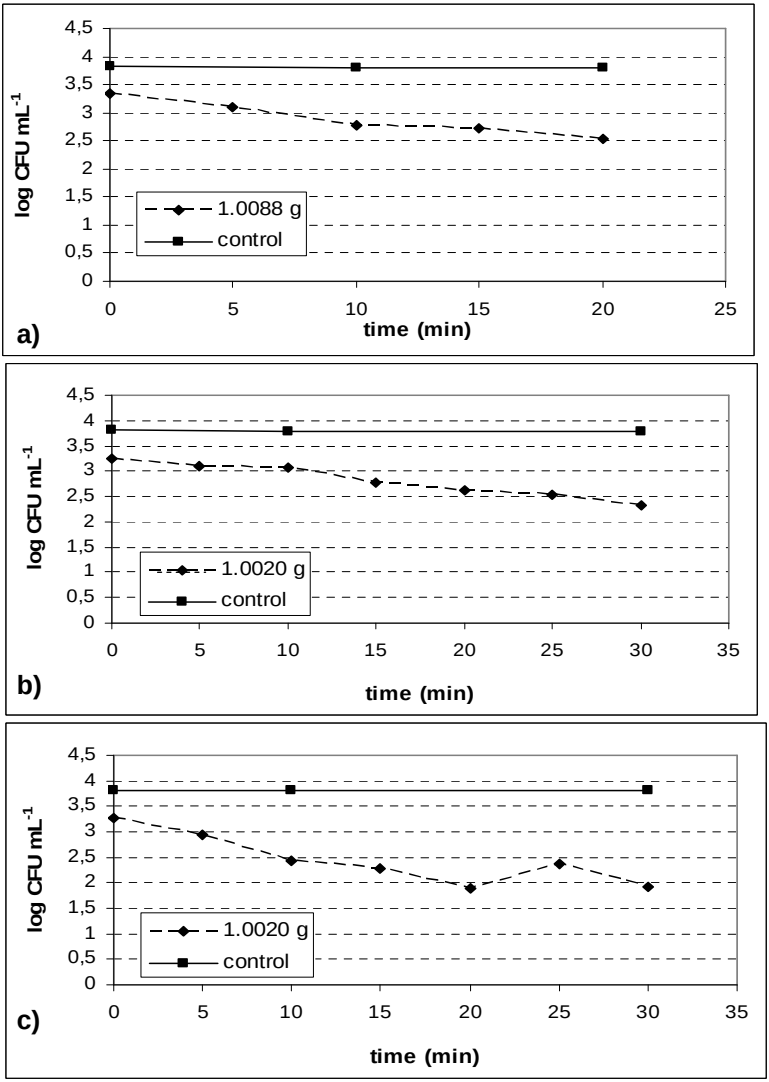

Figure 3(a-c) - *Antimicrobial activity of CF on the effluent of a wastewater plant.*

7.4 Conclusions

The recovery of a waste product from a thin metallic silver films manufacturing company was proposed, by using it as antimicrobial agent. The silver flakes were treated with thermal activation in reducing atmosphere and chemical activation. The flakes obtained by the thermal activation process in reducing atmosphere were named reduced flakes (RF) and the flakes obtained by the chemical activation process were named chemical flakes (CF).
We tested the antimicrobial activity of RF and CF in two systems in order to simulate possible applications: (1) against *E. coli* using tap water as culture medium and (2) against total heterotrophic bacteria of an effluent of a wastewater treatment plant.
The activated flakes reduced the cell density over time in both cases and their antimicrobial activity was affected by the experimental medium. The kill rate was lower in tap water and in the effluent than in deionised water.
The activated flakes proved their efficacy in the disinfection of real samples, also in the wastewater treatment plant effluent, where different bacteriological species were present. However, the time needed to reach zero microorganisms concentration was longer than the contact times required in the plants.
On the basis of the results with tap water, it can be hypothesised to use the activated silver flakes in household devices for drinking water purification and sterilisation, preventing the proliferation of bacteria in the filtering cartridge.

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Chapter 8

Conclusions

Pollution crisis is a major problem all around the world. It had adversely affected the lives of millions of people and caused many deaths and health disorder. Water pollution is one of the main causes for this crisis and majority of the water-borne diseases are spreading because of the poor quality of water, particularly due to the presence of bacteria and viruses in the water. Hence it is important to purify the water before its use [1]. There are several methods of water purification like chlorination [2], iodination [3, 4], ozonation [5], UV-purification [2], reverse osmosis [6]. However there are some disadvantages associated with these methods. For example the byproducts formed during chlorination are known as carcinogenic [7]. Recently many environmentally friendly inorganic bactericidal materials, including silver, have been studied for disinfection of water.

The antimicrobial effects of silver (Ag) have been known for thousand of years. In ancient times, it was used in water containers and to prevent putrefaction of liquids and foods. At the beginning of last century, Gibbard [8] was the first to systematically investigate the antimicrobial properties of silver. Many works study its use to inhibit the proliferation of microorganisms for medical [9], food packaging [10] and water treatment [11, 12] applications. However the exact mechanism of action of silver on the microbes is still not known but possible mechanisms of action have been suggested according to the morphological and structural changes found in the bacterial cells. Various techniques were used to study the mechanism of action and so the effect of silver on bacterial cells [7, 13, 14]. The antimicrobial activity of silver is usually dependent on the silver cations Ag^+, which bind strongly to electron donor groups in biological molecules containing sulphur, oxygen or nitrogen. Hence the silver-based antimicrobial materials have to release the Ag^+ to a pathogenic environment in order to be effective. The oxidation of the metallic silver to the active species Ag^+ was proposed to take place

through an interaction of the silver with the water [15]. Furthermore silver adsorbs oxygen onto the surface as atomic oxygen. Because atomic oxygen fits into the octahedral holes of the silver, oxygen atoms accumulate within the bulk of silver [16]. Some Authors attributed the bactericidal activity of silver to catalytic oxidation involving reactive oxygen species [17-20]. Silver catalytic activity was due to chemisorption of atomic oxygen on the surface of silver combined with freedom of movement of oxygen throughout the crystal lattice of the silver [21].
The aim of the research was the development of new products and processes from a manufacturing waste from a metallurgic Italian company which produces thin silver metallic films. Mainly we studied the possibility to use silver flakes in water disinfection processes. After a thorough survey of literature (chapter 2), regarding this we investigated following aspects:

- Antimicrobial activity of silver flakes before and after treatment with three activation processes (chapter 3);
- The silver flakes effect on the bacterial cells (chapter 4);
- The possible mechanism which gives the flakes antimicrobial property (chapter 5 and chapter 6);
- Some applications in real systems (chapter 7).

We concluded that only the activated flakes possessed antimicrobial capability. Furthermore the kill rate was dependent on the type of activation process, in fact the chemically activated flakes were the more efficient. The antimicrobial capability was dependent on flakes amount: increasing the flakes amount increased the removal rate. Furthermore the flakes maintained their properties also when re-used. Regarding the effect of the silver flakes on bacterial cells in aqueous medium, we showed that *E. coli* cells were damaged when treated with activated flakes: 10-20 % of the cells appeared damaged and

80-90 % dead at fluorescence microscopy observation. SEM photographs showed cells disruption and consequently death. So we classified the flakes as bactericidal agent.

The silver flakes released silver in water and the concentration increased with the contact time. Furthermore the Ag released by CF was greater than that released by RF and TF at each contact time. We hypothesised that the silver dissolved in water was in ionic form, so conducted antimicrobial tests in solutions of $AgNO_3$ as Ag^+ source. *Escherichia coli* in presence of silver ions decreased much faster than in presence of RF. Furthermore we used the eluate, obtained by filtration of RF immerged in water for 45 min, as medium for antimicrobial tests. We didn't detect any antimicrobial activity. On the basis of these results we concluded that the presence of flakes was necessary to kill microorganisms and the silver dissolved in water wasn't in ionic form. So the antimicrobial activity of the silver flakes didn't depend on the silver dissolved. Furthermore we characterized the flakes surface morphology and chemical composition. We observed by SEM technique that after the two thermal activation processes (in reducing atmosphere and in air) on the surface flakes grains with various dimensions appeared and the presence of dissolved oxygen was detected by XPS technique. We hypothesised that the antimicrobial activity was due to oxygen.

Finally we tested the antimicrobial activity of RF and CF in two systems in order to simulate possible applications: (1) against *E. coli* using tap water as culture medium and (2) against total heterotrophic bacteria of an effluent of a wastewater treatment plant.

The activated flakes reduced the cell density over time in both cases and their antimicrobial activity was affected by the experimental medium. The kill rate was lower in tap water and in the effluent than in deionised water.

The activated flakes proved their efficacy in the disinfection of real samples, also in the wastewater treatment plant effluent, where

different bacteriological species were present. However, the time needed to reach zero microorganisms concentration was longer than the contact times required in the plants.
On the basis of the results with tap water, it was proposed to use the activated silver flakes in household devices for drinking water purification and sterilisation, preventing the proliferation of bacteria in the filtering cartridge.

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THE FUNNY "COFFEE BREAKFAST"

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