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Bactericidal Performance of Chlorophyllin-Copper Hydrotalcite Compounds

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Abstract Copper hydrotalcites with and without adsorbed chlorophyllin exhibit a bactericidal effect that depends on the copper release and the basicity, which can be tuned through the chlorophyllin adsorption. The prepared solids performed well for the elimination of *Escherichia coli, Enterobacter aerogenes, Salmonella enterica*, and *Staphylococcus aureus* bacteria. The results showed that the copper-containing hydrotalcite with the adsorbed chlorophyllin is the most active material. Wastewaters from a metal industry were treated with these hybrid compounds, and the bactericidal effect was comparable with the results reported using more complex methods such as photocatalysis. Furthermore, one main advantage of these hybrid compounds is its low human toxicity compared with silver-containing materials.

The main highlights of this work are

- Microwave irradiation is an alternative method to obtain chlorophyllin/copper hydrotalcites.
- Copper-containing hydrotalcites has a bactericide effect.
- Copper disposal and hydrotalcite basicity determined the bactericidal activity.
- Chlorophyllin/copper hydrotalcite was most effective to treat wastewater.

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1 Introduction

Coliform bacteria are microorganisms that are commonly present in wastewater and can affect human health because they promote different types of diseases. Thus, the treatment of wastewater is imperative. However, physical methods, such as ultraviolet light or ionizing radiation, are very expensive. Chemical methods, such as ozone or chlorine and its derivatives, are often used, but their main disadvantage is that they form toxic and carcinogenic by-products (Poyatos et al. 2010; Song et al. 2014; Zhang et al. 2010). There are other novel alternatives, such as metal ions that are supported in natural and synthetic zeolites (De la Rosa-Gómez et al. 2008; Demirci et al. 2014), hydroxyapatites (Kim et al. 1998), or hydrotalcites (Sunayama et al. 2002). More recently, it has been shown that metallic nanoparticles exhibit efficient antibacterial properties due to their extremely large surface areas that provide a better contact with microorganisms (Qu et al. 2013; Rai et al. 2009). The structure of the metallic nanoparticles is also a determinant factor: the silver plane (111) exhibits a potent biocidal action (Gangadharan et al. 2010). However, in a large-scale process, the use of silver or nanoparticles may not be feasible due to their high cost. Copper, which is much cheaper than silver, is a valid option.

Copper ion has a high oligodynamic effect and thus, it acts as a strong bactericidal agent (Santo et al. 2008). The metal activity series against microflora in water, as reported by Albright and Wilson (1974), is: Ag > Cu > Ni > Ba > Cr > Hg > Zn > Na > Cd, showing that copper occupies the second position. It can be supported on zeolites or cationic clays where copper is found as an exchangeable cation or nanometric cluster, but copper may be lixiviated during the bacterial destruction process. Instead, hydrotalcites, which are anionic clays whose structure contains metal ions with a charge of 2+, may be prepared as a copper hydrotalcite (Cu-hydrotalcite). The advantage is that copper atoms are fully dispersed in the hydrotalcite structure. Furthermore, hydrotalcites are basic solids whose alkalinity should favor the bacterial destruction (Sunayama et al. 2002).

Hydrotalcites (HT) are also known as layered double hydroxides (LDH) and have the following general formula:

$$\left[M^{2+}{}_{1-x}M^{3+}{}_{x}(OH)_2\right](X^{m-})_{x/m}\cdot nH_2O$$

where M^{2+} is a divalent metal that may be replaced by a trivalent atom, M^{3+}, that produces positively charged layers. This charge is neutralized by X^{m-}, which is a compensating anion with charge $m-$ as in CO_3^{2-} or NO_3^{-}. The x represents the metal ratio $M^{3+}/(M^{3+}+M^{2+})$, and n is the number of water molecules (Fig. 1). The cation nature, the ratio of M^{3+}/M^{2+}, the synthesis method, and other factors (He et al. 2006) determine the hydrotalcite properties. Hydrotalcites, similar to many clays, may be expanded by introducing compounds between the layers through a process known as intercalation (Evans and Slade 2006). Hydrotalcites are often used as drug deliverers (Aguzzi et al. 2007). They are nontoxic and are often recommended as a stomach antiacid (Peterson et al. 1993). In Cu-hydrotalcite, the M^{2+} ions are magnesium or copper, which is a constituting atom of the hydrotalcite layers.

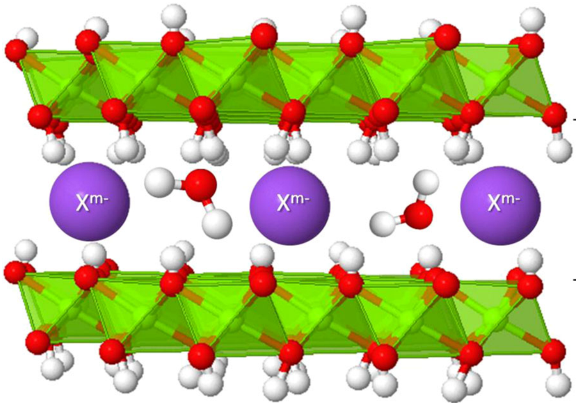

Fig. 1 Structural representation of hydrotalcites

Copper is also found in molecules, such as chlorophyllin (Chl), where it is linked to organic moieties. Chlorophyllin is a semi-synthetic derivative of chlorophyll, and its most common form is a sodium/copper chlorophyllin (Fig. 2). Unlike natural chlorophyll, chlorophyllin is water soluble (Sommer-Márquez et al. 2014). Chlorophyllin is the active ingredient used in the treatment of wounds, injuries, and other skin conditions, notably radiation burns. Moreover, chlorophyllin may be found in food additives (Tumolo and Lanfer-Márquez 2012). Chlorophyllin can be

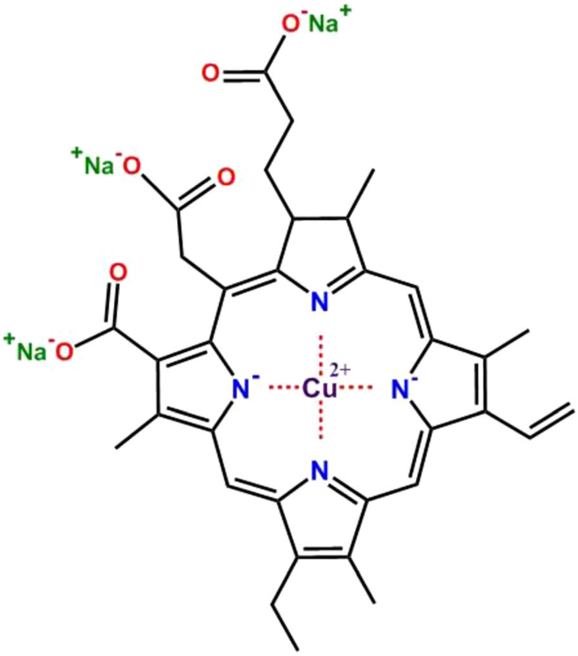

Fig. 2 Sodium/copper chlorophyllin structural formula

described as an anionic species, and it is easily retained in hydrotalcites, which are clays with basic properties (i.e., they have anion exchange sites). These clay properties promote the chlorophyllin stabilization and dispersion over the hydrotalcite surface (Sommer et al. 2013).

The antimicrobial activity is correlated with both the copper cations and the basicity of the materials (OH groups). Therefore, the formation of hybrids composed of chlorophyllin and Cu-hydrotalcite may provide copper in two different coordinations and in two different chemical environments. Moreover, chlorophyllin has a basic character, and when added to hydrotalcite, an increase in the bactericide activity could result. Thus, the copper disposal and the degree of basicity of the materials can determine the bactericidal performance.

Thus, the aims of this work were first to determine the influence of the basicity and the copper in different chemical environments (in chlorophyllin and/or in hydrotalcites) as antimicrobial agents against four microorganisms: *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, and *Salmonella enterica*. Second, we tested the bactericidal effect of the most active hydrotalcite sample in the treatment of a wastewater obtained from a metal industry.

2 Experimental

2.1 Synthesis

2.1.1 Materials

Magnesium (Sigma-Aldrich, 99 %), copper (Sigma-Aldrich, 99 %), and aluminum nitrate (Sigma-Aldrich, 98 %) were used as the reactants to synthesize the hydrotalcites. Sodium hydroxide (Baker, 99.5 %) was used as the precipitating agent. Chlorophyllin, a coppered tri-sodium salt, was provided by Sigma-Aldrich. A medium viscosity sodium alginate was purchased from Vetec Química Fina. Calcium chloride (>99 %) was provided by Merck.

2.1.2 Samples

MgAl Hydrotalcite This sample was synthesized by dropping in simultaneously a Mg- and Al-nitrate aqueous solution (2.5 mol L^{-1}) and a sodium hydroxide solution (2.0 mol L^{-1}) in which the flow of each solution was adjusted to maintain a constant pH of 9.0. The amounts correspond to a molar ratio of Mg/Al of 3.0. The resulting mixture was treated in a microwave autoclave (MIC-I, Sistemas y Equipos de Vidrio S.A. de C.V.) operating at 2.45 GHz for 10 min. The microwave equipment consists of a glass reactor of 500 mL, which fits in a microwave oven. An impeller-type stirring mechanism is adjusted to the reactor so that the temperature is the same all over, and it is controlled through an infrared radiation sensor. The temperature was fixed at 80 °C, and the power was fixed at 200 W. The solids were recovered by decantation, washed with distilled water to achieve a pH value of 8.0, and dried overnight in an oven at 70 °C.

CuMgAl Hydrotalcite This sample was prepared by simultaneously dropping a mixed aqueous solution of Cu, Mg, and Al nitrates (2.5 mol L^{-1}) with a sodium hydroxide solution (2.0 mol L^{-1}). The dropping flow of each solution was adjusted to maintain a constant pH of 9.0. The solution amounts were adjusted to correspond to a (Cu+Mg)/Al molar ratio of 3/1 in which the Cu:Mg molar proportion was 1:1. The resulting mixture was treated in a microwave autoclave at the same conditions as described above. The recovered solids were washed and dried in the same way as for the MgAl sample.

Chl-MgAl Hybrid This sample was synthesized as already described in our previous work (Sommer et al. 2013) by dropping simultaneously a Mg- and Al-nitrate solution (2.5 mol L^{-1}), a sodium hydroxide solution (2.0 mol L^{-1}), and a chlorophyllin dispersion that constituted 50 mg of chlorophyllin in 50 mL of distilled water. In our prior work, the synthesis was performed in undissolved chlorophyllin excess, whereas in the present work the amount of chlorophyllin is controlled. The pH was maintained constant at 9.0 by adjusting the dropping flow of each solution. The chlorophyllin amount corresponded to 5.0 mg g^{-1} of the hydrotalcite. The obtained slurry was microwave treated, washed, and dried using the same conditions as described above.

Chl-CuMgAl Hybrid This sample was synthesized similar to the previous CuMgAl sample, but the chlorophyllin dispersion was added dropwise to the mixture. The amount of each component was similar to the previous samples, and the synthesis conditions were the same as described above.

Alginate Composites Hydrotalcites or hybrid chlorophyllin-hydrotalcites consist of small particles (less than 2 μm) and cannot be evaluated as a bactericide using the inhibition haloes technique, which requires particles of ca. 5 mm diameter (Gangadharan et al. 2010; Díaz-Visurraga et al. 2012). They need to be dispersed in a microbiological inert matrix, such as alginate (protein chains forming a net), to form beads.

The chlorophyllin powder or the hydrotalcite samples were dispersed in deionized water (1.0 g/20 mL) and stirred for 24 h at room temperature. Then, 1.0 g of sodium alginate was added, and the mixture was again stirred for 24 h. A slurry was obtained that was then dropped on a 0.1 mol L^{-1} calcium chloride solution. The slurry was washed by stirring for 2 h with deionized water to eliminate all the chloride and sodium ions. With this procedure, a very sticky slurry (paste) of alginate composite was obtained.

2.2 Characterization Methods

2.2.1 X-ray Diffraction (XRD)

A Bruker D8 Discover diffractometer coupled to a copper anode (λ=1.54056 Å) X-ray tube and equipped with a Göbel mirror was used to obtain the X-ray diffraction patterns. Diffraction data were collected at room temperature in the Bragg-Brentano θ–2θ geometry. The scanning covered the 5°–70° range with a step angle of 0.025° and an integration time of 36 s.

2.2.2 Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES)

The Cu, Mg, and Al contents were measured in a 730-ES spectrometer from Varian. The samples (ca. 100 mg) were heated to 300 °C and then dissolved in a HNO_3–HCl (1/3 *v*/*v*) solution before analysis.

2.2.3 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra in the region of 4000–400 cm^{-1} were recorded with a Magna-IR Spectrometer 550 Nicolet. The sample was dispersed in pellets prepared with KBr.

2.2.4 Nitrogen Physisorption

N_2 adsorption-desorption isotherms were measured with a Micromeritics ASAP 2020 system at −198 °C. Prior to analysis, the samples were pre-treated in vacuum at 150 °C overnight. This pre-treatment ensures that the hydrotalcite is fully dehydrated and the structure is maintained. The pore size distribution was evaluated from the desorption branch of the isotherm using the BJH model.

2.2.5 Scanning Electron Microscopy (SEM)

A scanning electron microscope, Jeol JSM-6610LV, operating at 15 keV was used. The samples were covered with gold prior to analysis to avoid charging effects. The local elemental chemical composition was determined by energy-dispersive spectroscopy (EDS).

2.3 Bacteria

Four bacteria were investigated for antibacterial tests: three Gram negatives and one Gram positive. The Gram negatives were selected as fecal contamination indicators: *Escherichia coli* (ATCC–25922, Microbiologics, USA), *Enterobacter aerogenes* (ATCC–13048, Microbiologics, USA), and *Salmonella enterica* (ATCC–14028, Microbiologics, USA). The Gram positive was selected as an indicator of human influence in the water medium: *Staphylococcus aureus* (ATCC–25923, Microbiologics, USA).

2.4 Bactericide Tests

Firstly, a specific bacterial culture was diluted in a 0.1 % (*m*/*v*) peptone salt solution to reach the suspension turbidity at λ=565 nm of the value of 0.5 on the McFarland scale, which correspond to the bacteria concentration of 1.0×10^9 colony forming units per milliliter (CFU mL^{-1}). This solution was spread uniformly over the solidified nutrient agar gel (M1461, Himedia, Brazil) contained in a Petri dish. Then, a syringe (10 mL capacity) was used to insert the alginate composites over the plate to form bead-like (usually four) paste of ca. 5 mm diameter. The plate was then incubated in an oven at 37 °C for 2 days to measure the inhibition zone. The inhibition zone was evaluated by measuring the thickness of the transparent halo that formed around the bactericide bead paste (alginate composite) that was placed on the plate. The thickness of the halo was independent of the bead paste diameter. All samples were tested with the four bacteria.

The sample that exhibited the best bactericidal activity was selected to treat industrial wastewater. The sample was immersed in the wastewater by stirring for many hours. The composites formed by the alginate and hydrotalcites did not consist of rigid beads and thus could not be used for this purpose. Another preparation using a copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) as a matrix was performed. This copolymer is a biodegradable, nontoxic, biocompatible polymer that is naturally produced by bacteria. It has been reported that when this matrix is mixed with montmorillonite clay, it forms hard beads (Carli et al. 2011) and can be used in the treatment of wastewater without collapse of the beads.

The procedure was as follows: 50 g of PHBV (Y1000P grade) and 1.5 g of the sample, Chl-CuMgAl, were dried separately at 70 °C in vacuo. Then, they were mixed at 100 rpm in a rotatory chamber at 180 °C for 10 h. The mixture was then cooled to room temperature, resulting in a thick lamellar plastic-like material that was grinded in a knife mill to obtain particles with ca. 4.0–5.0 mm diameter. The resulting composite was labeled as Chl-CuMgAl/PHBV. The test was performed by mixing 200 mg of the grinded composite, Chl-CuMgAl/PHBV, with 100 mL of wastewater in an Erlenmeyer flask while stirring.

The metallurgy industry wastewater contained a 6100 colony-forming units per milliliter (CFU mL^{-1}) of total coliforms and 2700 CFU mL^{-1} of *Escherichia coli*.

3 Results

Our results are organized as follows. First, we present the characterization of the synthesized materials: the hydrotalcites with and without copper (CuMgAl and MgAl samples) and the composites (Chl-CuMgAl and Chl-MgAl). Chlorophyllin was also studied for comparison purposes. Then, the bactericidal performance of these five materials (chlorophyllin and the four hydrotalcite-based samples) was tested on model solutions of *Escherichia coli*, *Enterobacter aerogenes*, *Salmonella enterica* and *Staphylococcus aureus*. From these results, the best bactericide was chosen to treat industrial wastewaters that had a complex composition.

3.1 Characterization

3.1.1 Crystallinity of the Hydrotalcite-based Samples (XRD)

Figure 3 presents the XRD patterns of the hydrotalcite compounds showing that all samples are crystalline and may be identified as layered double hydroxides. No other crystalline compounds were observed. The first peak is located at d_{003}=8.6 Å, indicating that the interlayer anions are nitrates and carbonates. The doublet peaks, 110 and 130, that appeared at 61°–63° (2θ) in the MgAl sample indicate a regular order of the magnesium and aluminum cations in the hydrotalcite lamellae. The addition of copper and/or chlorophyllin molecules to the hydrotalcite causes a disorder in the interlayer region shown by the less defined 110 and 130 peaks (Ayala et al. 2011). The broadening of the peaks may be attributed either to the small particle size or to a strain between the crystalline planes (Blanch-Raga et al. 2013).

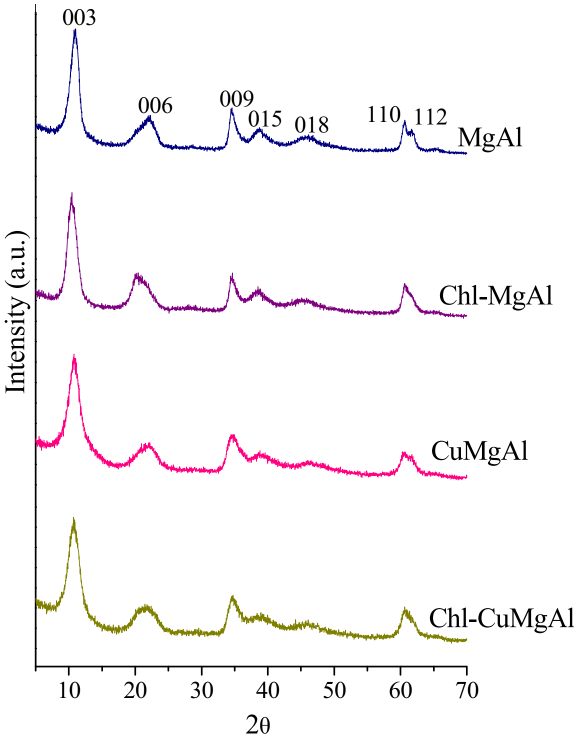

Fig. 3 X-ray diffraction patterns of the samples

3.1.2 Elemental Composition of the Hydrotalcite-based Samples (ICP-OES)

The experimental elemental composition was determined by the ICP-OES and shows molar ratio M^{2+}/M^{3+} values of 2.7, 2.8, and 2.8 for the MgAl, Chl-MgAl, and Chl-CuMgAl samples, respectively (Table 1). In the MgAl sample, the obtained values (Mg/Al=2.7) correspond within error range to the nominal ratios, ca. 3.0. When chlorophyllin was added during the synthesis for the Chl-MgAl sample, those values were reproduced. The copper content of chlorophyllin was not detected, although the sample was a green color. The CuMgAl sample contains 24.1 wt.% of copper, which is in a Mg/Cu ratio of 1.3 instead of the expected 1.5. A fraction of the initial copper present in the synthesis mixture must have been lost during the washing process after the hydrotalcite formation. Copper incorporates strongly to the hydrotalcite network, as previously shown by Sunayama et al. (2002). They obtained a ratio of Mg/Cu of 10, although they worked in copper excess (ca. ten times). This is consistent with an octahedron elongated by Jahn-Teller effects (Köckerling et al. 1997). The chlorophyllin did not interfere in the MgAl preparation, but when added to the CuMgAl reaction mixture, more copper was lost (ca. 40 %). In the Chl-CuMgAl sample, not only was the Jahn-Teller effect responsible for the copper loss, but the copper diffusion, which was limited by the chlorophyllin, may also be a determinant (Ayala et al. 2011). As the copper amount diminished, the amount of magnesium increased to maintain a constant Mg+Cu/Al molar ratio.

3.1.3 Functional Groups of Chlorophyllin (FTIR)

The chlorophyllin FTIR spectrum presents bands at 2800–3450 cm^{-1} and 1350–1450 cm^{-1} that correspond to CH_2-CH_2 groups. The bands at 1500–1700 cm^{-1} are due to C=N-C vibrations, and the small and broad bands appearing at 1650–1750 cm^{-1} can be attributed to ester radicals (H-CO-O-R) (Kim et al. 2002; Sommer et al. 2013). Unfortunately, the bands that were attributed to the main four functional groups of the hydrotalcite (Fig. 4) overlap those of chlorophyllin. They include the band at 3460 cm^{-1} attributed to the OH stretching vibrations in the lamellae, the band at 1640 cm^{-1} due to the water OH groups, the band at 1380 cm^{-1} attributed to the nitrates and carbonates and the bands between 420 and 833 cm^{-1} related to the Mg-O, Cu-O, and Al-O vibrations. Thus, it was not possible to determine the presence of the chlorophyllin adsorbed on the hydrotalcite using FTIR.

3.1.4 Specific Surface Area and Porosity of the Hydrotalcite-based Samples

The specific surface areas of the MgAl samples, displayed in Table 2, show that the addition of chlorophyllin did not modify the texture of the MgAl sample. The area was maintained (9–7 $m^2\ g^{-1}$) as well as the monomodal pore size distribution, which presents a peak at a 80–90 Å diameter. This result is in agreement with the previous XRD and ICP-OES results. The copper-containing hydrotalcite (CuMgAl) exhibits the highest specific surface area (42.8 $m^2\ g^{-1}$), which is more than four times the specific surface area of the MgAl sample. As mentioned, the Jahn-Teller effect predicts an elongation of the octahedra. Such stress favors the formation of smaller particles and, therefore, a higher specific surface area (Ayala et al. 2011). The pore size distribution was bimodal with a maximum at 35 and 300 Å. However, if chlorophyllin was added, less copper was incorporated and the Jahn-Teller effect was reduced. Consequently, the obtained material (Chl-CuMgAl) reproduced the previous values of the specific surface area but not the pore size distribution, which remained bimodal (peaks at 20 and 70 Å).

Table 1 Elemental composition obtained by ICP-OES and the resulting metallic molar ratios of the hydrotalcite samples

Sample	Mg (%)	Cu (%)	Al (%)	Mg/Al	Mg/Cu	Mg+Cu/Al
MgAl	24.7	0.0	10.0	2.7	-	2.7
Chl-MgAl	24.4	0.0	9.4	2.8	-	2.8
CuMgAl	11.9	24.1	9.9	1.3	1.3	2.4
Chl-CuMgAl	14.8	20.4	9.8	1.9	2.1	2.8

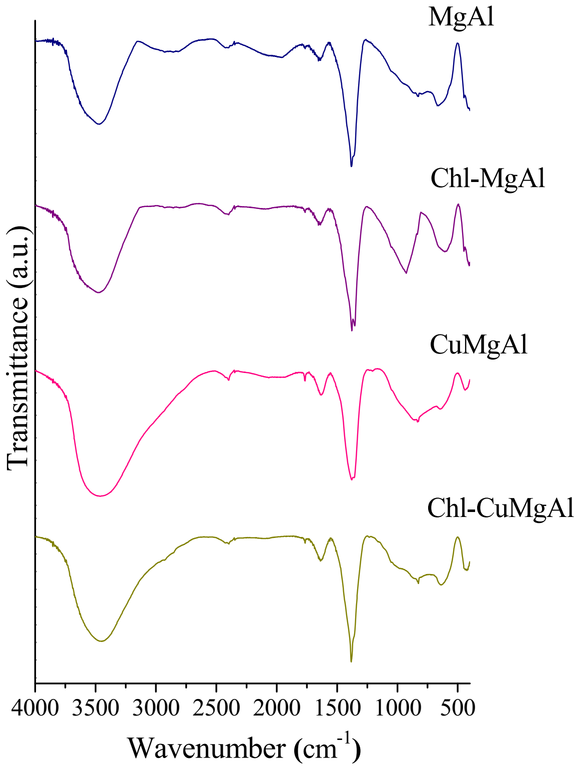

Fig. 4 FTIR spectra of the samples

3.1.5 Morphology of the Hydrotalcite-based Samples (SEM)

The morphology of the MgAl sample consisted of the features often reported, i.e., chunks between 0.5 and 10 μm with a rather rough surface (Fig. 5). If chlorophyllin was added, the chunk surfaces became smoother, and the particles became round. The size was less inhomogeneous at approximately 3.0–6.0 μm.

When the copper sample was prepared, the particles became globular and much smaller (200 nm). They were associated in an open network around pores less than 0.8 μm. Copper has both a disaggregating and a fragmenting effect. This observation is in agreement with the observed specific surface area reported previously. Thus, copper favors more crystallization nuclei then magnesium, and the addition of chlorophyllin inhibits this process. Hence, the hydrotalcite particles with less copper were expected. As already proposed, copper did not diffuse easily in the presence of chlorophyllin. Furthermore, the chlorophyllin acted as an agglomerant of the CuMgAl hydrotalcite particles.

Table 2 Comparison of the specific surface areas and pore size distributions of the hydrotalcite samples

Sample	Specific surface area (m^2 g^{-1})	Main peaks in the pore size distributions (Å)
MgAl	9.0	80
Chl-MgAl	7.3	90
CuMgAl	42.8	35 and 300
Chl-CuMgAl	8.3	20 and 70

3.1.6 Local Elemental Composition of the Hydrotalcite-based Samples (EDS)

The elemental surface amount of copper, as determined by EDS, in the samples is displayed in Table 3. When the MgAl and Chl-MgAl sample were compared, a very low amount of chlorophyllin was retained per Mg atom, as already shown by the Mg/Cu ratio obtained by the ICP-OES analysis. Instead, the CuMgAl and Chl-CuMgAl samples exhibited a Mg/Al ratio of 1.5 and 2.4, respectively, which reproduce within error the values already obtained by ICP-OES. In the Chl-CuMgAl sample, the copper amount is also due to the copper contained in the hydrotalcite and in the chlorophyllin. Because hydrotalcite is synthesized in the presence of chlorophyllin, copper (the heaviest ion) diffusion is inhibited by the large molecules of chlorophyllin, and the resulting hydrotalcite is magnesium enriched. This phenomenon was already reported for nickel/magnesium hydrotalcites (Rivera et al. 2007).

3.2 Microbiological Tests

3.2.1 Effect of Sample Composition on the Inhibition of Escherichia coli

The bead-like forms of hydrotalcite and alginate were evaluated using the Petri plate method (Gangadharan et al. 2010). Figure 6 shows the inhibition zones for *Escherichia coli* in the Chl, MgAl, Chl-MgAl, CuMgAl, and Chl-CuMgAl samples after 48 h of exposure. Chlorophyllin (Chl sample) was progressively liberated from the beads, and no halo was observed, indicating no bactericidal effect. The MgAl sample also was unreactive in the presence of *Escherichia coli*. Beads of Chl-MgAl showed one transparent and circular halo of 1.0 mm thickness (Table 4), corresponding to the inhibition of bacterial growth or bacterial destruction.

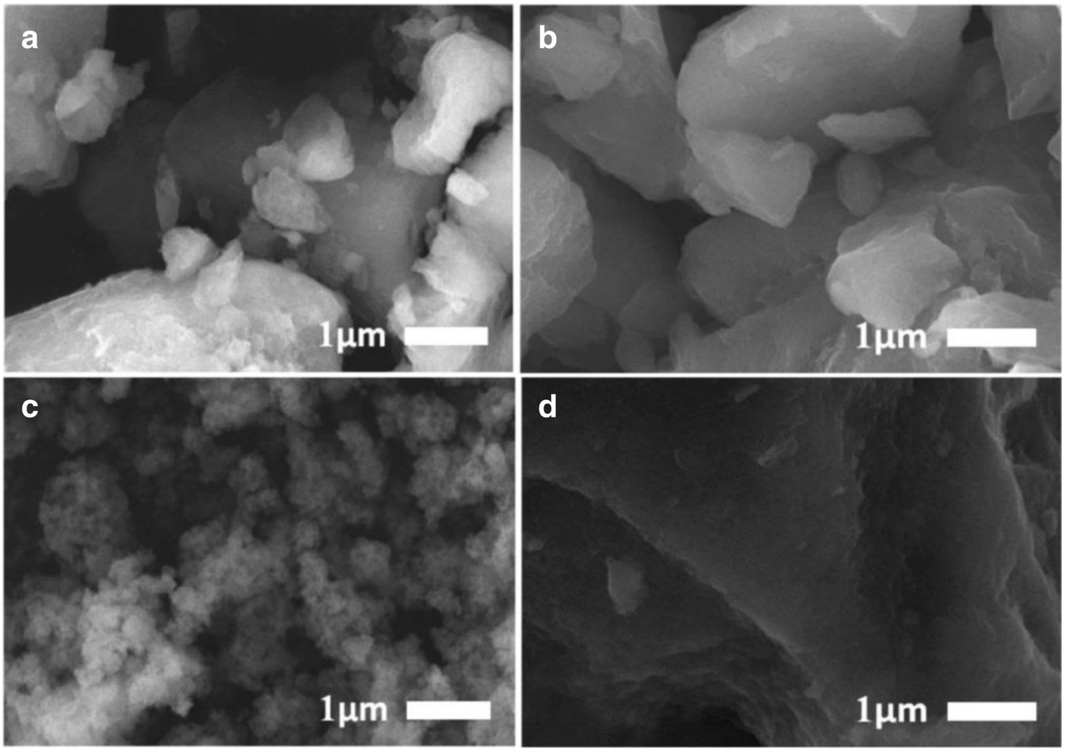

Fig. 5 SEM images of the hydrotalcite samples with and without chlorophyllin: MgAl(**a**), Chl-MgAl (**b**), CuMgAl (**c**), and Chl-CuMgAl (**d**). All at the same magnification (×10,000)

The CuMgAl and Chl-CuMgAl samples exhibited two concentric and transparent circular haloes around the initial beads of a 4.0 and 3.0 mm thickness and 6.0 and 3.0 mm thickness, respectively. The middle opaque halo that was attributed to bacterial presence in both of the samples was 1 mm thick.

3.2.2 Effect of Sample Composition on the Inhibition of Enterobacter aerogenes, Salmonella enterica and Staphylococcus aureus Growth

Samples were also tested with *Enterobacter aerogenes*, *Salmonella enterica* and *Staphylococcus aureus* bacteria. The results are summarized in Table 4. The pure chlorophyllin, the Chl sample, did not present any antimicrobial activity for the three bacteria because no inhibition halo was formed. To show the differences in the halo shapes depending on the bacteria, the results obtained with the Chl-MgAl sample and the selected bacteria are presented in Fig. 7.

Table 3 Surface elemental composition determined by EDS and the resulting metallic molar ratio of the hydrotalcite samples

Sample	Mg (%)	Cu (%)	Mg/Cu
MgAl	57.0	-	-
Chl-MgAl	59.2	0.38	155.8
CuMgAl	39.8	26.7	1.5
Chl-CuMgAl	48.0	20.2	2.4

For *Enterobacter aerogenes*, the inhibition halo was not circular and was a fried-egg shape in all of the active samples. For this bacterium, all of the hydrotalcite compounds exhibited an antimicrobial activity, even the MgAl sample, which presented a 2.0 mm halo. The halo increased to 3.0 mm for CuMgAl and reached 10.0 mm for the samples containing chlorophyllin, Chl-MgAl, and Chl-CuMgAl.

For *Salmonella enterica*, the haloes formed were similar in shape to those observed for *E. aerogenes*. In this case, no bactericidal activity was observed for the MgAl sample. Instead, haloes thickness of 3.0, 4.0, and 8.0 mm were observed for the Chl-MgAl, CuMgAl, and Chl-CuMgAl samples, respectively.

For the *Staphylococcus aureus* bacteria, only the samples containing chlorophyllin (Chl-MgAl and Chl-

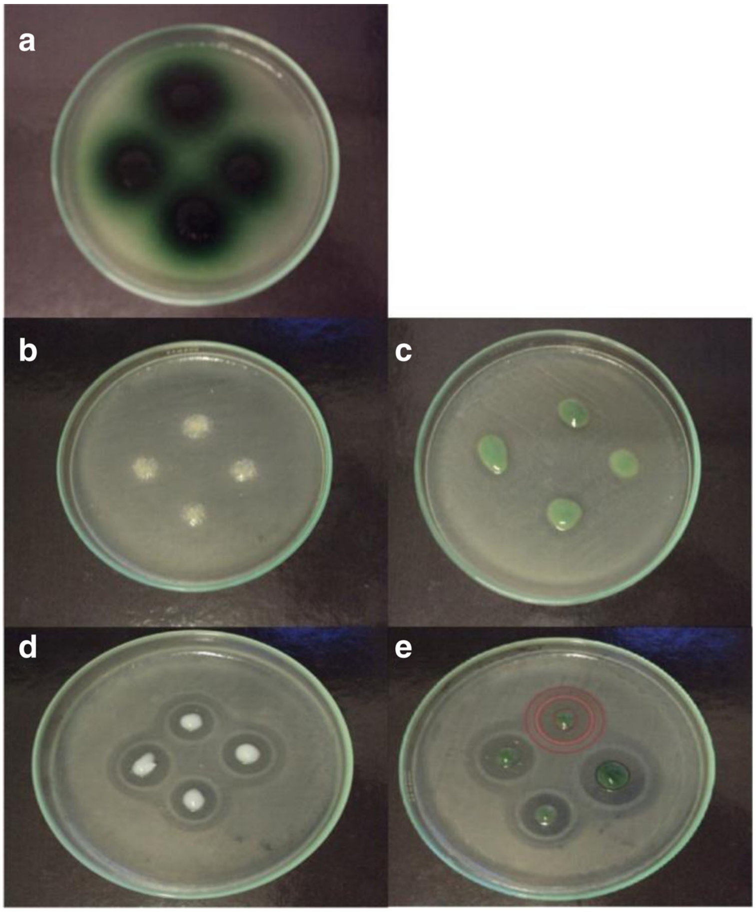

Fig. 6 Inhibition zones for *Escherichia coli* after a contact time of 48 h with the samples: Chl (**a**), MgAl (**b**), Chl-MgAl (**c**), CuMgAl (**d**), and Chl-CuMgAl (**e**)

CuMgAl) exhibited bactericidal activity. A small inhibition zone (0.5 mm) was observed for the Chl-MgAl sample, and a 3.0 mm zone was observed for Chl-CuMgAl.

3.2.3 Time Effect on the Inhibition of Escherichia coli Growth with the Chl-CuMgAl Sample

The Chl-CuMgAl sample inhibited the growth of all of the bacteria; thus, it was selected for the study of its bactericide activity as a function of time against *Escherichia coli*. The evolution of the circular inhibition haloes with time (5, 25, and 48 h) is shown in Fig. 8. The number of the haloes increased with time. At 5 h, only one transparent halo was observed, and at 25 h, a second transparent halo appeared. After 48 h, the second transparent halo thickness increased. After 48 h, the system remained stable, and the haloes did not evolve.

Table 4 Halo thicknesses, in millimeters, defined as the external halo minus the internal halo radii

Sample	*Escherichia coli*[*]	*Enterobacter aerogenes*	*Salmonella enterica*	*Staphylococcus aureus*
Chl	0.0	0.0	0.0	0.0
MgAl	0.0	2.0	0.0	0.0
Chl-MgAl	1.0	10.0	3.0	0.5
CuMgAl	*4.0*;1.0;*3.0*	3.0	4.0	0.0
Chl-CuMgAl	*6.0*;1.0;*3.0*	10.0	8.0	3.0

[*] When several haloes are present, the thickness of each one is reported, including transparent (italicized numbers) and opaque haloes

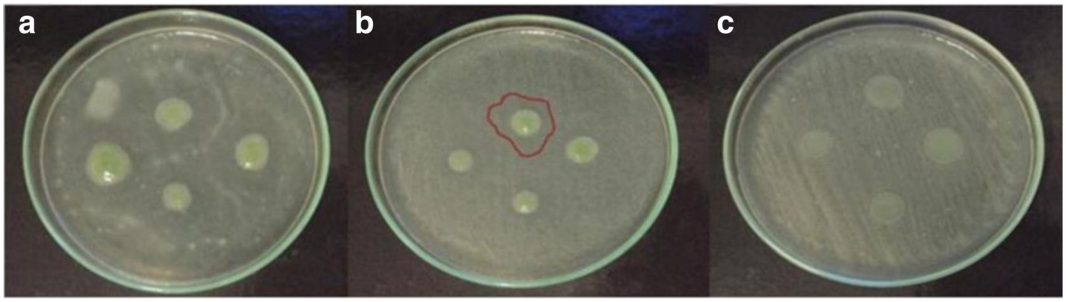

Fig. 7 Inhibition zones observed with sample Chl-MgAl for *Enterobacter aerogenes* (**a**), *Salmonella enterica* (**b**), and *Staphylococcus aureus* (**c**)

3.3 Industrial Wastewater Bactericidal Treatment Test

The Chl-CuMgAl sample, which was the best performing as shown previously, was supported on PHBV and tested to treat a metallurgic industry wastewater. Figure 9 shows the bactericidal activity for the total coliform and for the *Escherichia coli*. The number of total coliforms decreased rapidly from 6100 to 4000 CFU mL^{-1} in 60 min, and it then decreased slowly and linearly to 3000 CFU mL^{-1} in 360 min. The reduction in the number of colonies corresponds to 35 % in 1 h and 51 % in 6 h.

For *Escherichia coli*, a similar bactericide behavior was observed. The initial 2700 CFU mL^{-1} was reduced to 1400 CFU mL^{-1} in 60 min (26 %) to reach 800 CFU mL^{-1} after 6 h, which corresponds to a 70 % bacterial elimination. If the percentages of the killed coliforms and *Escherichia coli* are compared, *Escherichia coli* was more easily attacked. The difference was as much as 20 %, although the initial bacteria concentrations were different.

4 Discussion

Our results are summarized as follows. All samples were well crystallized, and no other compounds were observed. The incorporation of copper into the hydrotalcite lattice generated so much stress in the crystalline network that the particles were small. When chlorophyllin was incorporated into the synthesis procedure for both the samples with or without copper, the particles were smooth and consisted of large agglomerates. Because chlorophyllin is a well-known basic compound and it forms anionic species in solution, the basicity of the resulting hybrid hydrotalcite was expected to be higher. The same characteristic explains why chlorophyllin agglomerates the small hydrotalcite particles together. This chlorophyllin effect was confirmed by the low content of copper in the Chl-CuMgAl sample. Indeed, the large chlorophyllin volume obstructed the copper diffusion.

MgAl hydrotalcite was selectively active against *Enterobacter aerogenes*, showing that a low basicity is enough to eliminate this bacterium. The same sample was inactive against all other microorganisms. This result was essential to understand how the hydrotalcite-containing samples perform.

When the performance of the MgAl and CuMgAl samples were compared, the copper effect was most evident. Against all Gram-negative bacteria (*Escherichia coli, Enterobacter aerogenes*, and *Salmonella enterica*), the copper-containing sample was more active, primarily against *Escherichia coli.*

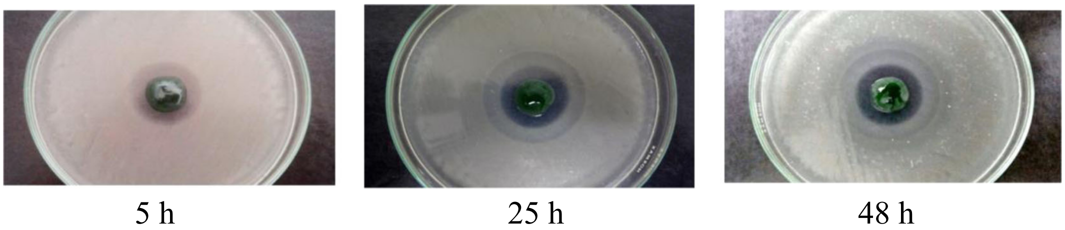

Fig. 8 Evolution of the inhibition halos with time for the sample Chl-CuMgAl in contact with *Escherichia coli*

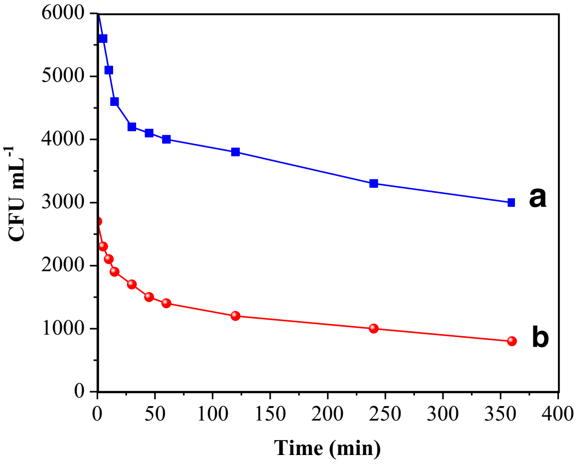

Fig. 9 Bactericidal activity of sample Chl-CuMgAl/PHBV in the purification of wastewater from a metallurgic industry. The survival of total coliforms (**a**) and *Escherichia coli* (**b**) is expressed as CFU mL^{-1} of wastewater

Copper seems to migrate from the structure and reacts as a cation-free moiety to interact with the bacteria. When chlorophyllin was added (MgAl compared with Chl-MgAl and CuMgAl compared with Chl-CuMgAl), the basicity was increased, and the bactericide effect also increased, even for the Gram-positive *Staphylococcus aureus*. Hence, the microorganism destruction may be correlated with the copper content and the increased basicity due to the addition of chlorophyllin.

An increasing number of haloes were observed as a function of time, for the *Escherichia coli* experiment, which may be related to a bacteriostatic effect due to the progressive and noncontinuous release of copper from the material. Copper that was released may have attacked a large number of bacteria to produce the first halo. Then, hydrotalcite was encapsulated by the dead microorganisms, and copper had to diffuse not only from hydrotalcite but also across the shell. As copper diffused progressively, a second halo formed. The effect was enhanced by chlorophyllin (i.e., by a higher basicity) as the first ring thickness increased from 4.0 to 6.0 mm. This mechanism could explain the evolution with time.

The Chl-CuMgAl sample was selected due to its high bactericidal efficiency to treat industrial wastewaters. The efficiency at low times was comparable to the results obtained with more complex methods, such as photocatalysis (Alena and Sahu 2013; Song et al. 2014) or with metal compounds (Santo et al. 2008; Kawahara et al. 2000). This material may be a promising choice for water bactericidal treatment and microbial control. It is a solid that exhibits low human toxicity and a low cost. Therefore, to avoid bacterial contamination, this hybrid material could be incorporated into many everyday products, such as ceramics or even textiles.

5 Conclusion

Copper is an essential metal that was included in the composition of the bactericidal hydrotalcites, which act via two mechanisms: basicity and copper release. The basicity may be enhanced by adsorbing chlorophyllin, and the copper release depends on the bacteriostatic effect, primarily for *Escherichia coli* growth inhibition. After testing on *Escherichia coli*, *Enterobacter aerogenes*, *Salmonella enterica*, and *Staphylococcus aureus,* the best sample composition was found to be a chlorophyllin adsorbed on a copper-containing hydrotalcite. This sample was most effective in the bactericidal treatment of industrial wastewater and may be recommended for the incorporation into everyday products.

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