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Organic biocides hosted in layered double hydroxides: enhancing antimicrobial activity

Hybrid organic-inorganic biocides

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Abstract: Samples of layered double hydroxides containing carbonates as compensating anions were prepared by the urea method. These LDHs were used as hosts of anions coming from pipemidic and nalidixic acid. XRD results confirm that these anions were hosted in the interlayer space of LDHs. Further, from ^{27}Al NMR MAS characterization of an interaction between the brucite-like layers and anions was suggested. Then the hybrids LDHs were used as biocide of *Salmonella typhi* and *Escherichia coli*. The release profile of pipemidic and nalidixic anions from hybrid LDHs occurs for periods as long as 3.5 hours. The free-organic acid LDHs were not able to kill *S. Typhi*, neither *E. coli*. In contrast, the hybrids LDHs eliminate almost completely bacteria within short times.

Keywords: Adsorption; Escherichia coli; Salmonella typhi; Biocides; Layered Double Hydroxides.

1 Introduction

In human society the control mechanisms to inhibit the bacteria proliferation are often not efficient, conversely microbial infections are one of the main killers in the world [1]. The treatment of microbial infections becomes more and more complex because of the number of resistant microbial strains plus that antibiotic immune patients grow significantly faster than the number of useable antibiotics [2,3]. Thus, antimicrobial agents are required to control pathogen growth. Efficiency of antimicrobial materials is determined by several physicochemical factors [4-6]. Most commonly bactericides are metal [7,8] for instance copper, gold and silver are well known to be efficient when they are sized at nanoscale [9,10]. Other biocides, however, are toxic, which makes them undesirable for applications in sensitive media such as drinking water, foods or textiles. A lot of organic molecules also have potential to be applied to the control bacterial growth [11,12] but sometimes their use is restricted because they are easily dissolved and they could react before they reach the cell membrane. A way to potentiate the biocide effect of organic molecules is to incorporate them into an inorganic material in order to assure they reach the bacteria. This approach has been applied successfully for other organic molecules with a biological function such as antioxidants [13], glucose sensor [14] and enzymatic processes [15], among others. Taking into account these examples, a good hybrid material with biocide properties could emerge from the combination of nalidixic or pipemidic acids (chemical structure of anions of these two acids is shown in figure 1) as the organic biocide and the layered double hydroxides as inorganic shields.

Nalidixic acid acts, at low concentration, is a bacteriostatic agent, which means that it inhibits bacterial growth but at high concentration it becomes a bactericide, in other words, it is able to kill bacteria [16]. Pipemidic acid is also a biocide agent that acts mainly against the gram-negative bacteria [17].

Layered double hydroxides (LDHs), often known as hydrotalcite-type compounds can be described by the formula $[M^{II}_{1-x}M^{III}_{x}(OH)_2]^{x+}(A^{n-}_{x/n})\cdot mH_2O$. M^{II} and M^{III} are divalent and trivalent cations which are bonded to

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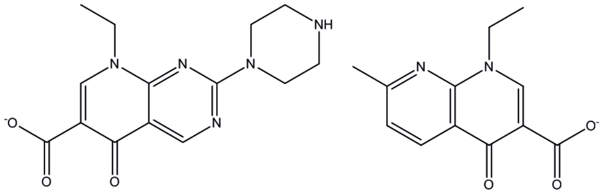

Figure 1: Chemical structures of anions of pipemidic acid (left) and nalidixic acid (right).

six OH groups to form octaedra [$M(OH)_6$] which in turn are sharing edges to build brucite-like layers. In layers of LDHs a partial substitution of the divalent cations by trivalent ones occurs and consequently a positive charge is induced in the layer; compensation of this positive charge is made by anions located between the brucite-like layers, these anions are solvated with water molecules [18] in the interlayer space. The chemical composition of LDHs drives their physicochemical properties, thus a great collection of LDHs has been synthesized, varying mainly the nature of trivalent and divalent cations but also diversifying the anions. Commonly, simple inorganic anions such as carbonate, phosphate, halides, or nitrate are the compensating anions in LDHs [19] but organic anions can also be found in LDHs. Synthesis of LDHs containing three or more cations in the layers [20] is also possible.

A unique characteristic of these layered materials is the called memory effect which is their ability to rebuild their layered structure, lost on heating at a moderate temperature (400-500°C), when exposed to water and anion containing solutions [21].

LDHs have found applications, mainly as base catalysts for many organic reactions and in minor amounts as vectors to guide organic and biologic molecules [22,23]. Thus, taking advantage of LDH properties and pipemidic and nalidixic acids, we started this work with the goal to prepare efficient hybrid biocide materials against *E. coli* and *S. typhi*.

2 Experimental procedure

2.1 Synthesis

LDHs were synthesized by the urea hydrolysis method [24]. As source of Zn and Al the salts $Zn(NO_3)_2{\bullet}6H_2O$ and $Al(NO_3)_3{\bullet}9H_2O$ were used, respectively. The synthesized LDH was dried at 120°C for 12 h. This dried solid was labelled LDH-CO_3.

LDHs containing organic biocide were obtained from the carbonated one. 0.5 g of LDH- CO_3 was thermally treated at 400°C for 5 h under N_2 flux. The thermal-treated LDH was suspended in 30 mL of a solution containing sodium salts coming from pipemidic or nalidixic acid and then pH was adjusted to 9 by adding NaOH 0.1 M. The suspension was shaken for 7 days and after that the solid was recovered, washed and dried at 50°C. LDHs containing biocide were labelled LDH-CO_3-PIP and LDH-CO_3-NAD for solids with pipemidic or nalidixic acid, respectively. Chemical composition of LDH samples is reported in Table 1.

2.2 Characterization

The layered double hydroxides were characterized by X-ray diffraction (XRD), solid-state nuclear magnetic resonance, infrared spectroscopy (FTIR- ATR) and N_2 adsorption.

The XRD patterns were acquired using a diffractometer D8 Advance-Bruker equipped with a copper anode X-ray tube. The crystalline phases were identified by fitting the diffraction patterns with the corresponding Joint Committee Powder Diffraction Standards (JCPDS cards).

Infrared spectra were recorded in the ATR-FTIR mode using a Perkin-Elmer series spectrophotometer model 6X.

Solid state nuclear magnetic resonance (MAS NMR) measurements were performed in an Avance-400 Bruker (9.39 T) spectrometer. Chemical shifts were referenced to TMS. Solid-state ^{27}Al and ^{13}C magic angle spinning-nuclear magnetic resonance (MAS-NMR) experiments were performed on a Bruker Avance II spectrometer at frequencies of 104.2 and 100.58 MHz, respectively. ^{13}C CP MAS-NMR spectra were acquired using a 4-mm cross-polarisation (CP) MAS probe spinning at a rate of 5 kHz. Typical ^{13}C CP MAS-NMR conditions for the ^{1}H-^{13}C polarization experiment, included a $\pi/2$ pulse of 4 μs, contact time of 1 ms and delay time of 5 s. Chemical shifts were referenced to TMS. ^{27}Al MAS-NMR spectra were acquired using short single pulses ($\pi/12$) and a delay time of 0.5 s. The samples were spun at 10 kHz, and the chemical shifts were referenced to an aqueous 1 M $AlCl_3$ solution.

2.3 Bacteria experiments

Bacteria were acquired from Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional, Mexico. *E. coli* and *S. thyphi* strain (ATCC 25922) were grown in tripticaseine broth. For the growth experiments, a starter culture of each strain was inoculated with fresh colonies

Table 1: Chemical composition of hybrid LDH samples.

Code Sample	*Chemical formula*	Zn/Al ratio
ZnAl-CO_3	$[Zn_{0.661}Al_{0.214}(OH)_2](CO_3)_{0.107}0.89H_2O$	3
ZnAl- CO_3-PIP	$[Zn_{0.731}Al_{0.240}(OH)_2](CO_3)_{0.025}(PIP)_{0.192}0.81H_2O$	3
ZnAl- CO_3-NAD	$[Zn_{0.677}Al_{0.220}(OH)_2](CO_3)_{0.030}(NAD)_{0.158}0.77H_2O$	3

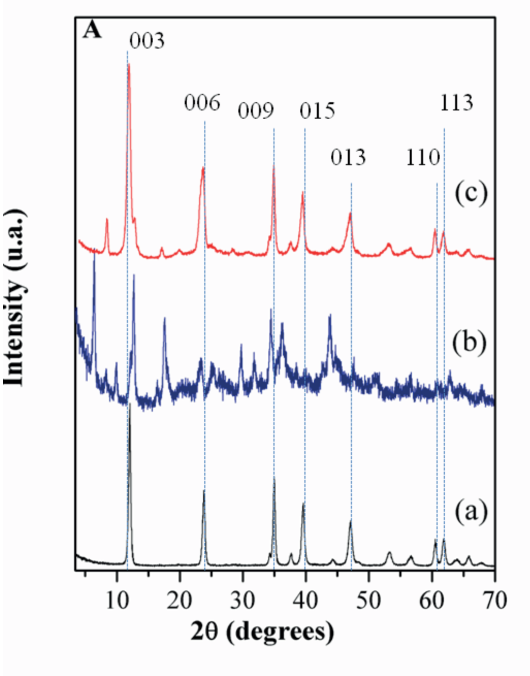

Figure 2: X-ray diffraction patterns of LDH-CO_3: (a) free of organic biocide, (b) containing pipemidic acid, and (c) containing nalidixic acid. As a reference, the lines with Miller index of hydrotalcite phase are included.

and incubated for 24 h in Tripticaseine medium. Bacterial growth rates were determined by counting the number of surviving colonies in a selective agar. Fresh medium (10 mL) was inoculated in test tubes with the starter culture and grown at 37°C with continuous agitation at 30 rpm. 2.5 mg of free biocide and the amount required of LDH-biocide to have a similar amount of organic biocide were then added to the culture, and the colonies were measured over a time course. The LDHs were recovered, dried and then used in a second experiment with bacteria. As reference, the bacterial growth rates were also performed in the presence of free biocides. During all experiments treated with bacteria the material used was sterilized and LDH was used immediately after preparation. Experiments were done in triplicate in order to have statistical significance.
Ethical approval: The conducted research is not related to either human or animals use.

3 Results

3.1 Materials

In Figure 2 are compared the XRD patterns of LDHs without or loaded with biocide organic molecules. XRD patterns of samples free of biocide exhibit a peak with Miller index (003) at 11.57 degrees corresponding to an interlayer distance (d_{003}) of 7.64 Å. XRD patterns shows evidence that incorporation of pipemidic acid in LDH-CO_3 leads to a loss of crystallinity. In the diffractogram of the LDH-CO_3-PIP the presence of a peak at low values of two-theta, close to 6.3 degrees, proves that distance between the layers increased. However, the peak with a d_{003} of 7.64 Å is also observed in LDH-CO_3-PIP. The XRD pattern of LDH-CO_3-NAD corresponds to LDH more crystalline than that observed for LDH-CO_3-PIP. In XRD pattern of LDH-CO_3-NAD the peak labelled (003) is also shifted to lower values if compared to LDH-CO_3 suggesting an intercalation of nalidixic anions between the brucite-like layers. XRD pattern of LDH-CO_3-NAD exhibits three peaks at 8.43°, 17.13° and 25.12° (2θ) which is indicative of larger distances between brucite-like layers. The peak at 8.43 degrees corresponds to a distance d_{003} of 10.48 Å. On the other hand, the peaks at 17.13° and 25.12° match with harmonics indexed (006) and (009), respectively.

FTIR spectra of LDH-CO_3 and biocide-LDHs are displayed in Figure 3. The spectrum of free biocide LDH sample presents the broad band centered at 3445 cm^{-1}, which is assigned to stretching (ν) of O-H bonds. The band due to the bending (δ) vibration of water molecules is found close to 1620 cm^{-1} for both samples. The band assigned to stretching C-O bonds coming from CO_3^{2-} species appears very intense at 1363 cm^{-1}. Lastly, a broad and asymmetric band observed is observed at wave number below 1000 cm^{-1}, which is assigned to vibration of metal-oxygen bonds present in brucite-like layers.

In the spectrum of LDH-CO_3-PIP the absorption bands due to main functional groups of pipemidic acid are observed. The band at 1635 cm^{-1} in the spectrum of LDH-CO_3-PIP is assigned to ν_{sym} of carboxilate group [25-27].

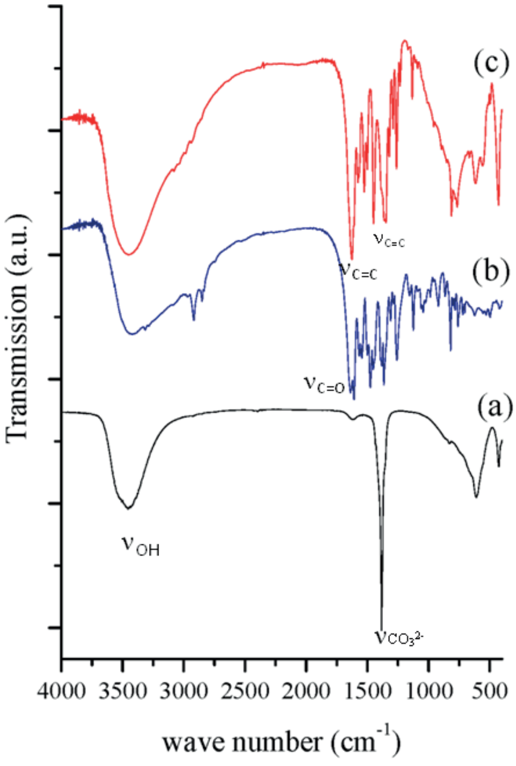

Figure 3: FTIR spectra of LDH-CO_3: (a) free of organic biocide, (b) containing pipemidic acid, and (c) containing nalidixic acid.

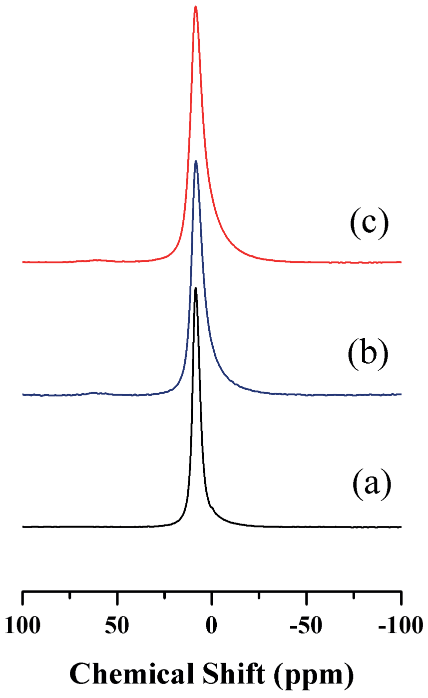

Figure 4: ^{27}Al MAS NMR spectra of LDH-CO_3: (a) free of organic biocide, (b) containing pipemidic acid, and (c) containing nalidixic acid.

The band at 1550 cm^{-1} is due to the stretching mode of C=O [28].

The spectrum of LDH- CO_3-NAD also shows evidence of the presence of nalidixic acid anions in LDH. In the spectrum of LDH-CO_3-NAD, the absorption band at 1463 cm^{-1} corresponds to ν_{sym} C=C of the aromatic rings, the band at 1496 cm^{-1} is due to ν of C=N. Lastly, the bands at 1536 and 1347 cm^{-1} are assigned to ν_{asym} and ν_{sym} of COO$^-$, respectively.

^{27}Al MAS NMR spectra displayed in Figure 4 show that coordination number of aluminium does not change with the biocide loading. In the spectrum of LDH-CO_3 only an isotropic peak close to 8 ppm is observed, which is due to aluminium six-fold coordinated to oxygen atoms [29]. When the LDH is loaded with biocide the peak becomes broader. ^{13}C CP-MAS NMR spectra, Figure 5, are in line with the ^{27}Al NMR results. The NMR signal of aliphatic carbons for both nalidixic and pipemidic acid are found in the range of 20-80 ppm and that of aromatic carbons within 90-175 ppm [30]. The spectra of pure sodium salts (spectra a and d) are composed of narrow well-defined peaks. In contrast, when organic molecules are incorporated to LDH, signals become broader, particularly those due to aromatic carbons.

3.2 Biocide activity

Figure 6 compares the curves of the number of *E. coli* colonies survived as time went on in the culture media and in the presence of ZnAl-CO_3-biocide materials. As reference samples, the free biocide and LDH without organic biocide were also tested. In the presence of ZnAl-CO_3 the number of colonies increases always and after 90 minutes the number of colonies was increased by 70%.

When the biocides are dissolved in culture media, as free biocide, they are efficient in killing 40% of the colonies for times as short as 30 min but after that the killing rate decreases significantly, and for the period from 30 to 120 min only 20% and 27% of the bacteria are abated by pipemidic and nalidixic acid, respectively.

The biocides intercalated in LDHs behave similar to free biocides in the first short period of 30 minutes, killing 46% of colonies. However, contrary to free biocides, both NAD and PIP intercalated in LDH continue to kill a high percentage of colonies at times longer than 30 minutes. Indeed, both biocide-LDH practically kill 85% of colonies in 95 minutes.

The release profiles of PIP and NAD from ZnAl-CO_3-biocide in 50 mM phosphate buffer at pH 7.5 are shown in Figure 7. The release profiles of NAD and PIP are very close

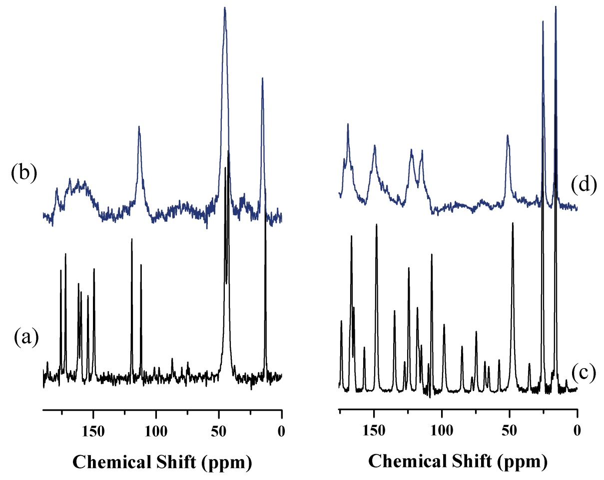

Figure 5: ^{13}C CP MAS NMR Spectra of (a) sodium pipemidic salt, (b) LDH-CO_3-PIP, (c) sodium nalidixic salt and (d) LDH-CO_3-NAD.

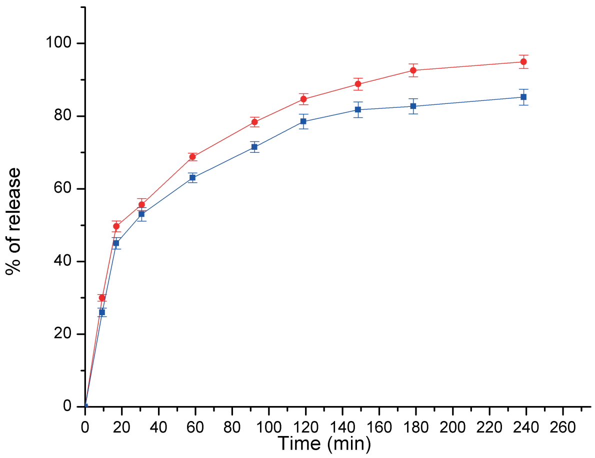

Figure 7: Release profile of NAD from LDH-CO_3-NAD (-■-) and PIP from LDH-CO_3-PIP (-●-) in 50 mM phosphate buffer at pH 7.5. The amount of NAD or PIP was monitored by UV-vis spectroscopy.

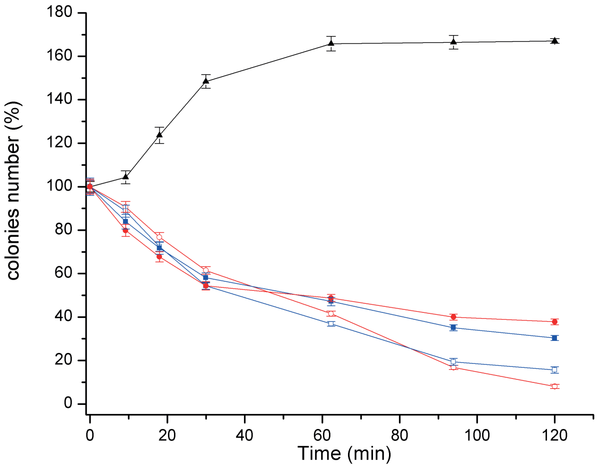

Figure 6: Evolution as a function of time of number of *E coli* colonies survived in culture media in the presence of PIP (-●-), NAD (-■-), ZnAl- CO_3 (–▲–), ZnAl- CO_3-NAD (–□–), ZnAl- CO_3-PIP (–○–).

in the first two hours, releasing close to 80% of biocide. For times longer than 2 hours the profile of LDH-CO_3-NAD is lower than that of LDH-CO_3-PIP but both materials release biocide after 2 h and at periods as long as 4 h almost the total biocide was leached from LDH-CO_3-PIP. In other words, after 3.5 hours the hybrid LDHs still contain either PIP or NAD, which can be useful to kill bacteria in a second experiment. Coming back to experiments with bacteria, in a first experiment, the maximal percentage of killed bacteria was 86 and 93% in the case of LDH-CO_3-NAD and LDH-CO_3-PIP, respectively. The biocide-LDH was recovered after experiments reported in Figure 6 and reused as bactericide in a second experiment, and the results are shown in Figure 8. The reused material is still bactericidal but their power to kill bacteria is lower than fresh materials, which should be related to the low remaining biocide after the first cycle as bactericide.

In Figure 9 is plotted the number of *S typhi* colonies survived as time went on in the culture media and in the presence of LDH-CO_3, organic biocides as well as in the presence of ZnAl-CO_3-biocide materials. In the presence of LDH-CO_3, nobacteriostatic or bactericide effect was observed and the number of colonies increased rapidly with time, and after 2 hours the number of colonies was nearlyduplicated. Free biocides are bactericides in the first 30 minutes, killing only 30% of the colonies; for longer times the bactericide effect diminishes and after 2 hours 55% of the colonies survive.

In the first 30 minutes, LDH-biocide materials behave similarly as bactericide of *S. thypi* to that observed for *E. coli*. After 30 minutes in the presence of ZnAl-CO_3-biocide materials, the number of colonies had diminished to 42 % of initial bacteria. The most interesting results is that observed after 30 minutes, both ZnAl-CO_3-NAD and ZnAl-CO_3-PIP continue abating colonies to reach a 72% killing efficiency which is almost 30% more than free biocides.

4 Discussion

The XRD results provides evidence that pipemidic and nalidixic anions where intercalated in LDH-CO_3 but with pipemidic acid the intercalation is accompanied with a loss of crystallinity suggesting that stacking of brucite-like

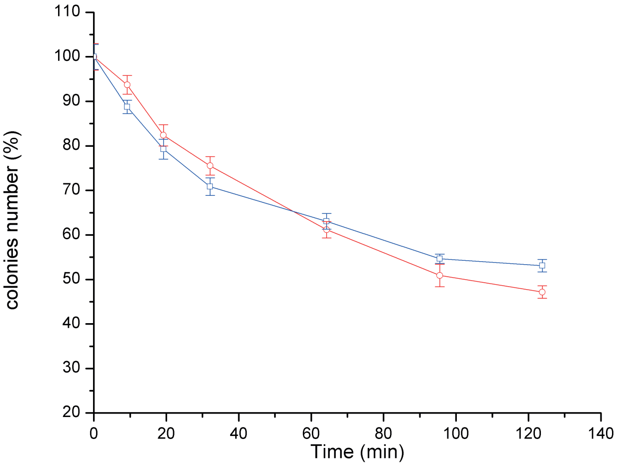

Figure 8: Evolution as a function of time of number of *E coli* colonies survived in culture media in the presence of reused LDH-biocide ZnAl- CO$_3$-NAD (–□–) and ZnAl- CO$_3$-PIP (–○–).

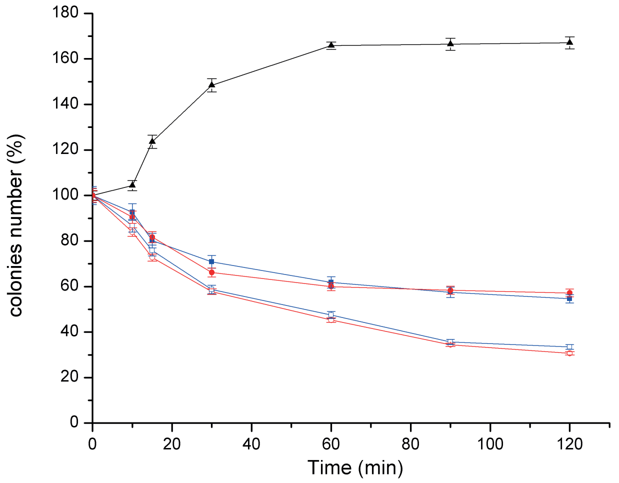

Figure 9: Evolution as a function of time of number of *S typhi* colonies survived in culture media in the presence of PIP (-●-), NAD (-■-), ZnAl- CO$_3$ (–▲–), ZnAl- CO$_3$-NAD (–□–), ZnAl- CO$_3$-PIP.

layers is less ordered. In both cases, organic anions do not totally replace the carbonate anions. The FTIR and NMR results suggest an interaction between brucite-like layers and organics anions. Actually, when the LDHs are loaded with biocide, the ^{27}Al MAS NMR peaks become broader suggesting that relaxation of the signal is affected most probably because of the presence of a large pi electron density coming from aromatic rings of the biocide.

The behaviour of the biocide-LDHs as antimicrobial agent against *E. coli* suggested that the system acts as a delivery vehicle of organic biocide and can be used for long periods. On the contrary, free biocide, without support, act as a bactericide at times as short as 30 minutes but after that it has only bacteriostatic properties.

The prolonged release of biocide from biocide-LDHs should be attributed to dipolar interactions between organic biocide and LDHs and occurs in other similar hybrid organic-LDHs composites [31,32]

Even though *S. typhi* is more resistant than *E. coli* to both acid nalidixic and pipemidic acids incorporated to LDHs, it is clear that the interactions of LDH-biocide are favourable to kill *S. typhi*. This could be related to the bactericidal mechanism of bacteria, which in general implies disruption of the cell wall by destabilising cations associated with the cell envelope. Thus, the orientation of biocide molecules through LDH interactions, and long period release could be an additional helpful agent in elimination of bacteria.

5 Conclusion

Pipemidic and nalidixic acid hosted in layered double hydroxides zinc-aluminium can be used as biocide materials of *Escherichia coli* and *Salmonela typhi*. Layered double hydroxides containing carbonates as compensating anions, without organic acids, are not able to kill bacteria. When biocides are incorporated to the LDHs, they become more efficient as bactericides, and are able to kill up to 90 and 70% of *E. coli* and *S. typhi*, respectively. In general, *S. typhi* shows more resistance to be killed by biocide materials.

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