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Performance of a Combined Bacteria/Zeolite Permeable Barrier on the Rehabilitation of Wastewater Containing Atrazine and Heavy Metals

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Abstract: Several chemicals, such as pesticides and heavy metals, are frequently encountered together in environment matrices, becoming a priority concerning the prevention of their emissions, as well as their removal from the environment. In this sense, this work aimed to evaluate the effectiveness of a permeable biosorbent bio-barrier reactor (PBR) on the removal of atrazine and heavy metals (copper and zinc) from aqueous solutions. The permeable bio-barrier was built with a bacterial biofilm of *R. viscosum* supported on 13X zeolite. One of the aims of this work is the investigation of the toxic effects of atrazine, copper and zinc on the bacterial growth, as well as the assessment of their ability to adapt to repeated exposure to contaminants and to degrade atrazine. The growth of *R. viscosum* was not affected by concentrations of atrazine bellow 7 mg/L. However, copper and zinc in binary solutions were able to inhibit the growth of bacteria for all the concentrations tested (5 to 40 mg/L). The pre-acclimation of the bacteria to the contaminants allowed for an increase of 50% of the bacterial growth. Biodegradation tests showed that 35% of atrazine was removed/degraded, revealing that this herbicide is a recalcitrant compound that is hard to degrade by pure cultures. The development of a PBR with *R. viscosum* supported on zeolite was successfully performed and the removal rates were 85% for copper, 95% for zinc and 25% for atrazine, showing the potential of the sustainable and low-cost technology herein proposed.

Keywords: permeable bio-barrier reactor; atrazine; heavy metals; zeolite; *R. viscosum*

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1. Introduction

The contamination of environmental matrices has increased exponentially in the past decades due to intense and growing industrial activity [1]. The contamination of aquatic matrices is of particular concern, not only for human health but also for all living beings, since a wide variety of pollutants may not only provoke acute or chronic effects, depending on the exposure duration, but also can be bioaccumulated, thus entering the food chain [2].

Considering characteristics such as toxicity, carcinogenicity and persistence, some chemicals have been considered as priorities, being thus subjected to monitoring and more restrictive legislation. As part of the Water Framework Directive, an European priority list of substances posing a threat to or via the aquatic environment was established [3]. The Directive 2013/39/EU includes a list of 45 chemicals, 14 of them are pesticides, namely triazine herbicides (atrazine and simazine), diuron herbicide and DDT insecticide.

Atrazine (2–chloro–4–ethylamine–6–isopropylamine–s-triazine) is an herbicide of the class of triazines, presented as a white crystalline solid, with a chemical structure represented by a triazine ring replaced with chlorine, ethylamine and isopropyl amine, which makes it recalcitrant for biological degradation in the environment. Atrazine is used worldwide for pre- and post-emergent control of invasive broadleaf and grassy plants in agriculture, especially for the cultivation of corn, sorghum and sugar cane, being the second most widely consumed pesticide in the world, with an annual consumption of

about 70,000–90,000 tons [4]. Atrazine has been included in the list of priority substances by the European Union and its use amongst EU countries was banned since 2004 [5]. However, many countries, including Brazil, U.S.A and China, continue to use it as one of the main agents to combat the growth of weeds in monocultures [6]. Even after two decades of prohibition within the European Union, the concentration of atrazine in groundwater remained close to 0.1 μg/L, which confirms its persistent character [7].

Heavy metals constitute another group of important contaminants due to their well-known toxicity, persistence in the environment and bioaccumulative nature. Different industries, such as leather tanning, electroplating, steel production, chemical and textile manufacturing, mining, etc., are the major sources of heavy metals in the environment [8].

Several treatment methodologies and processes have already been applied to rehabilitate environmental matrices polluted with several classes of pollutants including heavy metals, pharmaceuticals, pesticides, organic compounds, dyes, among others. The development of alternative treatment technologies has been rising, with particularly emphasis on biological and eco-friendly strategies [9]. The use of adsorbents such as clays and zeolites has been reported as a low-cost and effective alternative for the removal of several types of contaminants [10–14]. Zeolites are crystalline aluminosilicates with three-dimensional framework structures with high adsorption capacities and high internal and external surface areas. Permeable barriers constructed with both natural and synthetic zeolites have been used to remove heavy metals and other pollutants from water [15–19]. In parallel with sorption processes, biological treatments offer a cost effective and eco-friendly alternative to conventional rehabilitation methods. Several experiments conducted with microorganisms such as bacteria and/or fungi revealed their great potential for the bioremoval of toxic compounds [20–24]. Biofilms are bacterial communities in which cells are surrounded in a matrix that provides a beneficial structure with a higher biological activity and sorption area. This physical matrix allows a better protection against the harmful compounds and stressful conditions of the extracellular environment [25]. Due to their ability to entrap a wide range of inorganic and organic contaminants [26,27], biofilm communities perform an important and decisive role in the fate and transport of contaminants though environmental matrices [28,29].

The use of a combined system (microorganisms coupled with adsorbent materials) to rehabilitate aquatic matrices has proven its efficiency and eco-friendly character [26,30–33]. In this context, the concept of permeable bio-barrier is a promising and widely accepted rehabilitation technology for contaminated aquatic matrices [34]. This concept has been rising from lab to full-scale. The targeted contaminants are either immobilized, sorbed and/or degraded into less hazardous forms due to the wide range of biological and physico-chemical reactions that occur in the solid support [35]. This work aims to enable the development of a permeable biosorbent barrier using a *Rhizobium viscosum* biofilm supported on 13X zeolite, as well as the evaluation of its efficiency on the treatment of aquatic matrices contaminated with the herbicide atrazine and heavy metals (Cu and Zn).

2. Materials and Methods

2.1. Materials

The strain *Rhizobium viscosum* CECT 908, previously classified as *Arthrobacter viscosus* [36] was purchased from the Spanish Type Culture Collection, University of Valencia. All the growth medium nutrients were of analytical grade: peptone (Riedel), yeast extract (Fluka), malt extract (Fluka) and glucose (Riedel). The aqueous solutions of atrazine (Pestanal), $CuCl_2$ and $ZnCl_2$ (Panreac) were prepared by dilution in deionized water. Synthetic 13X zeolite was used as adsorbent material and support for bacterial growth. The zeolite 13X was supplied in the form of pellets (5–8 mm) by Xiamen Zhongzhao Imp. & Exp. Co. (Xiamen, China). Prior to use, the zeolite was autoclaved at 121 °C for 20 min.

2.2. Microorganism Growth

The culture medium used for the growth of bacteria was composed by glucose 10 g/L; malt extract 3 g/L, peptone 5 g/L and yeast extract 3 g/L. The optimal growth pH was adjusted to 7.0 [11]. Erlenmeyer flasks containing 250 mL of culture medium were inoculated with a pre-culture of *R. viscosum* CECT 908 and incubated at 26 °C under moderate agitation (150 rpm).

2.3. Analytical Procedures

An UHPLC Shimadzu Nexera X2 (Kyoto, Japan) equipped with a diode array detector was used for the quantification of atrazine. Separation was performed on a kinetex C18 column using a mobile phase consisting of a mixture of water/acetonitrile (55:45) (*v*/*v*) with a flow rate of 0.2 mL/min at 25 °C. The detection wavelength was adjusted at 225 nm, the injection volume was 5 µL and the autosampler was operated at 4 °C. A calibration curve was constructed over the concentration range 0.15–10.0 mg/L and was used to establish the linearity of the method. A 20 mg/L stock solution of atrazine was used for the preparation of the standard solutions. Each standard was analyzed in replicate, being the average peak areas used for quantification. The method detection limit and quantification limit were, respectively, 0.05 mg/L and 0.16 mg/L.

During batch and PBR assays, the concentration values of Cu and Zn were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). The operating conditions were the following: 8 L/min of argon plasma flow, 1300 W of radio frequency power, 0.2 L/min of auxiliary gas flow and 0.5 L/min of nebulizer gas flow. A multi-element ICP standard solution with a concentration of 1 g/L was used to prepare the calibration solutions. Prior to analysis, the samples were filtered and acidified with concentrated nitric acid (HNO_3 69%, Fisher). Periodically, the multi-element ICP QC standard solution (CHEM LAB) and a blank (HNO_3 2%) were used to test the instrument response.

2.4. Characterization Procedures

Scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FT-IR) were used to characterize the starting 13X zeolite and the samples obtained after biosorption experiments and PBR assays.

Approximately 1 mg of each sample was ground with 100 mg KBr in an agate mortar, and then, the pellet was obtained from the mixture by applying pressure. All spectra were obtained in the range 4000–500 cm^{-1} with a minimum of 30 scans and a spectral resolution of 4 cm^{-1}. Background correction for atmospheric air was performed during analysis.

2.5. Toxicity Assessment

Several toxicity experiments were performed, aiming to infer, in a first stage, about the capacity of *R. viscosum* to remove, actively or passively, several pollutants such as atrazine, copper (Cu) or zinc (Zn) when present as single pollutants or in mixture. In a second stage, similar experiments were performed using a pre-acclimated *R. viscosum* culture previously grown in a medium containing 2 mg/L of atrazine, 2 mg/L of Cu and 5 mg/L of Zn.

For all toxicity experiments, the first step consisted in adding 20 mL of a pre-inoculum of *R. viscosum*, previously cultivated in proper culture medium (see Section 2.2) and left for 24 h at 150 rpm and 26 °C to achieve the exponential growth phase. For the atrazine toxicity experiments, 20 mL of *R. viscosum* pre-inoculum was used and added to 250 mL Erlenmeyers flasks containing sterilized culture medium and different initial concentrations of that pollutant (1 mg/L, 3 mg/L, 5 mg/L and 7 mg/L) and left for 30 h, at 150 rpm and 26 °C. For Cu and Zn toxicity assessments, the *R. viscosum* pre-inoculum was added to 250 mL Erlenmeyers flasks containing sterilized culture medium and different concentrations of Cu and Zn (5 mg/L Cu and 10 mg/L Zn; 10 mg/L Cu and 20 mg/L Zn; 20 mg/L Cu and 40 mg/L Zn) and left for the same period of time and experimental conditions.

For both experiments, several samples (1 mL) were collected through time. These samples were centrifuged at 13400 rpm for 10 min and resuspended in 3 mL of H_2O.

The optical density (OD) of the suspension was read at 620 nm in a spectrophotometer (T60 UV—Visible Spectrophotometer, PG instruments). The biomass concentration was assessed through a calibration curve previously built.

For the tertiary assays (atrazine mixed with Cu and Zn) the first inoculum was added to solutions containing 2 mg/L of atrazine and different metal concentrations (5 mg/L Cu and 10 mg/L Zn; 10 mg/L Cu and 20 mg/L Zn; 20 mg/L Cu and 40 mg/L Zn; 30 mg/L Cu and 60 mg/L Zn). After 24 h, 1 mL sample was collected, and the OD was read in order to determine the biomass concentration obtained.

For the toxicity experiments with previous pre-acclimation, the same operational conditions were employed. The acclimation process consisted of inoculating *R. viscosum* in sterilized culture medium containing 2 mg/L of atrazine, 2 mg/L of Cu and 5 mg/L of Zn, which was kept in an incubator for 24 h at 150 rpm and 26 °C. A sample of 1 mL was collected and the OD was measured in order to determine the biomass concentration achieved and posteriorly used in the different sets of these toxicity experiments.

A control experiment was performed to compare the bacterial growth in the presence and in the absence of the contaminants. All tests were performed in duplicate and the results presented are an average of duplicates.

2.6. Biodegradation of Atrazine

R. viscosum was inoculated and grown for 24 h at 26 °C and 150 rpm in 500 mL of culture medium (see Section 2.2). After 24 h, 150 mL of this pre-inoculum was transferred to new culture media (100 mL) and left in an incubator for 48 h in the same operational conditions. After this period, the biomass was centrifuged for 11 min. The biomass pellets were re-suspended in distilled and sterilized H_2O to obtain a final biomass concentration of 4.0 g biomass/L.

The re-suspended pellets were then added to Erlenmeyers flasks containing different concentrations of atrazine (1 mg/L, 3 mg/L e 5 mg/L) and left at 150 rpm, 26 °C for 9 days. At different time intervals, 1 mL samples were collected, centrifuged at 13,400 rpm for 10 min. The supernatant was used for the estimation of atrazine concentration by UHPLC, whereas the pellet, after being re-suspended in 3 mL of distilled H_2O was used to determine the biomass concentration along time. A control was performed without biomass. The assays were conducted in duplicate and the presented results are an average of duplicates.

2.7. Biosorption of Atrazine and Heavy Metals

As in the biodegradation assays, in the biosorption experiments *R. viscosum* was inoculated and allowed to grow for 24 h at 26 °C and 150 rpm in 500 mL of proper culture medium (see Section 2.2). 15 mL of this pre-inoculum was transferred to new Erlenmeyer flasks containing 5 g of zeolite 13X, 150 mL of atrazine 1 mg/L, 40 mg/L of Cu and 60 mg/L of Zn. The Erlenmeyer flasks were kept under moderate stirring at 26 °C until equilibrium was reached. Several samples (1 mL) were collected over time and centrifuged at 13,400 rpm for 10 min. The supernatant was used to determined atrazine, Cu and Zn concentration in solution over time, whereas the pellet was used to measure the concentration of bacteria. The stock solutions of the contaminants were diluted in 100 mM acetic acid buffer at pH 4.7 in order to avoid the formation of metal hydroxides in solution. Control tests using only zeolite were also performed.

2.8. Permeable Bio-Barrier Reactor

A compact polycarbonate acrylic lab-scale permeable bio-barrier reactor (PBR) was used, consisting of a horizontal Plexiglas column (40 cm length, 15 cm Ø) with a bio-barrier made of bacterial biofilm supported on zeolite previously prepared (Figure 1). For the biofilm preparation an Erlenmeyer flask containing 400 mL of sterilized culture medium was inoculated with *R. viscosum* and left in an orbital incubator (150 rpm, 26 °C) for 24 h in order to achieve the exponential growth phase. Posteriorly, 100 mL of the pre-inoculum was transferred to Erlenmeyer flasks containing 1000 mL of sterilized culture medium along

with 500 g of 13X zeolite, which remained under moderate agitation (80 rpm, 26 °C) for 120 h to promote the adherence of the biomass to the support. After biofilm development, the zeolite was transferred and putted in the bio-barrier section of the reactor. The passage of the aqueous solution of atrazine (1 mg/L), Cu (26 mg/L) and Zn (40 mg/L) with a flow rate of 15 mL/min was promoted, passing thus through the column in recirculation. Samples were collected at the exit of the system over time and subsequently analyzed by ICP and UHPLC, to determine the concentration of metals and atrazine, respectively. The pH values of the flux before and after the bio-barrier were monitored over time.

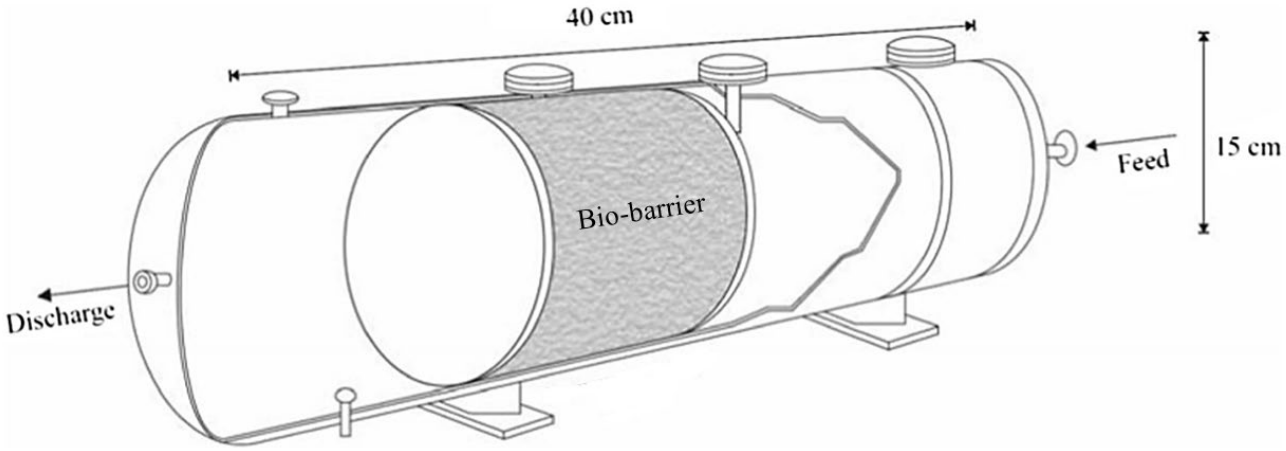

Figure 1. Permeable bio-barrier reactor design.

3. Results and Discussion

3.1. Toxicity of Atrazine

In order to evaluate the growth of *R. viscosum* in the presence of atrazine, toxicity tests were carried out according to the methodology described in Section 2.5. From these experiments, it was possible to construct the growth curves (Figure 2) at different initial concentrations of atrazine.

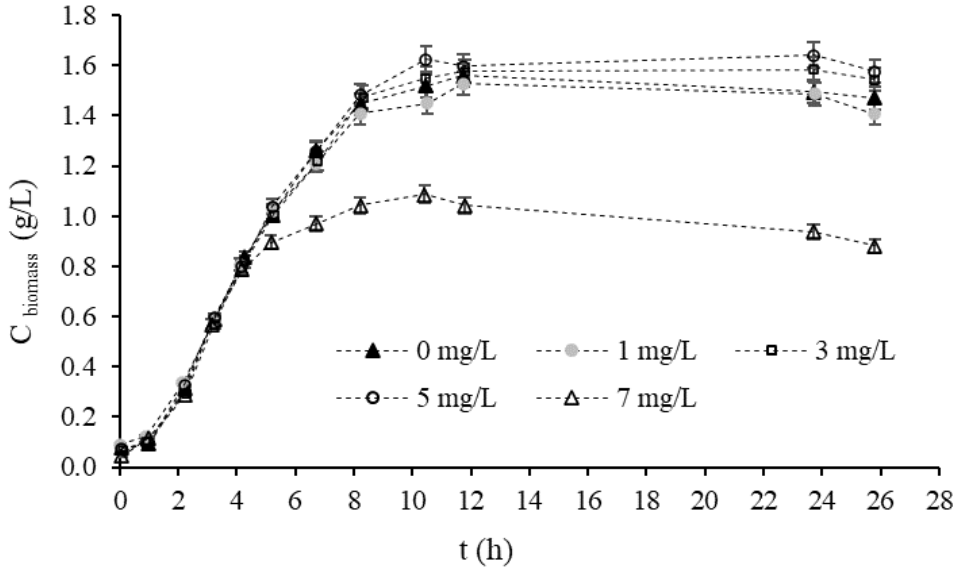

Figure 2. Toxicity assessment of atrazine. Biomass concentration along time for different initial concentrations of atrazine.

The results revealed that *R. viscosum* presented a typical growth behavior in the presence of atrazine, with initial concentrations up to 5 mg/L, while for the highest concentration (7 mg/L), a reduction in the length of the exponential phase was observed.

This finding can be attributed to the inhibitory effect of the herbicide, as demonstrated by Fang et al. [37] and Godoi et al. [38] who found a reduction in the microbial diversity of the soil after the application of different concentrations of atrazine, with the toxic effect becoming more significant as the amount of the herbicide applied increased. Despite the decrease in the growth of the culture exposed to the highest concentration of atrazine, the cultures with concentrations of herbicide up to 5 mg/L showed the same behavior as the control without atrazine. These results can be explained by the ability of this microorganism to use atrazine as a source of carbon, nitrogen and energy via enzymatic processes, where specific genes catalyze the hydrolysis process of the molecule [37,39,40], or simply because the herbicide does not interfere in its metabolic processes, as presented by Omotayo et al. [41]. These authors isolated two strains of the bacteria (Nocardioides EAA—3 and Nocardioides EAA—4) from agricultural soils contaminated with atrazine and reported that their metabolic capacity did not change during the different treatments carried out with different concentrations of herbicide. Thus, it is demonstrated that the bacteria studied in the present work has the ability to survive in environments contaminated with atrazine, a very important factor for the development of a biosorption technology.

3.2. Toxicity of Heavy Metals

The growth curve of *R. viscosum* was evaluated in the presence of Cu and Zn in binary mixtures with different initial concentrations. The results are shown in Figure 3. The range of concentrations used in these experiments was selected taking into account real values found in contaminated aquatic environments and agro-industrial sewers. The solution pH was not adjusted and varied between 6 (beginning of the assays) and 5 (after 24 h).

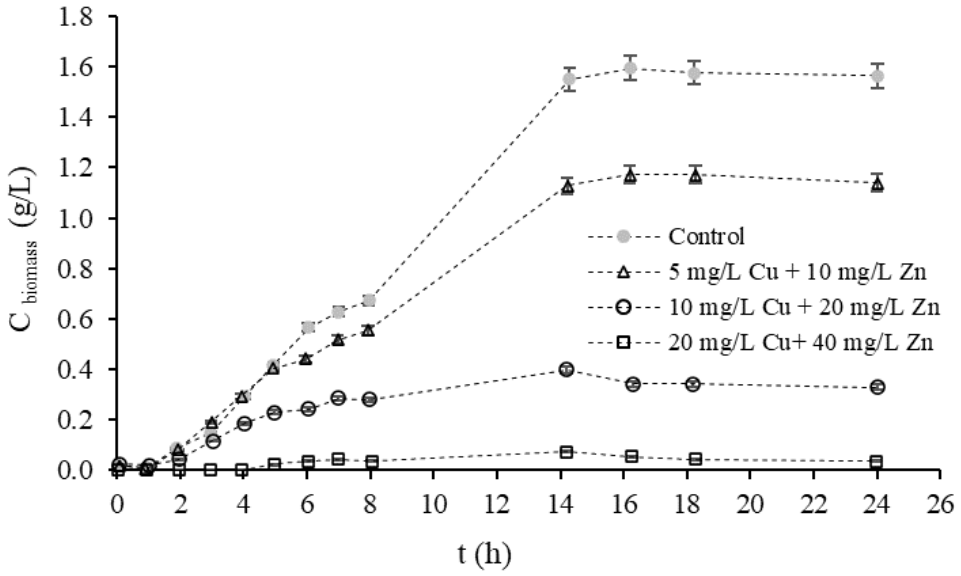

Figure 3. Toxicity assessment of heavy metals. Biomass concentration along time for different initial concentrations of Cu and Zn.

The results obtained show that Cu and Zn have a xenobiotic effect over *R. viscosum*, even in low concentrations (5 mg/L Cu and 10 mg/L Zn), with the growth being completely inhibited for the highest concentrations (20 mg/L Cu and 40 mg/L Zn). For the intermediate concentrations, a change in the "lag" phase was observed; the microbial culture undergoes intracellular adaptations and synthesizes new enzymes necessary for cell growth in the new environmental conditions. This had an impact on the other stages of microbial growth, since, as it was observed, the bacterium was not able to grow like the culture in the absence of heavy metals (control). For the lowest concentrations, the "lag" phase did not show any difference in comparison with the control, but a reduction of the exponential phase length was observed, possibly due to the toxic effect of the metals. Sengor and

Gikas [42] performed toxicity studies with copper and zinc at low concentrations (up to 0.65 mg/L) with *Arthrobacter* sp. and concluded that the metals stimulated the microbial growth separately, but when present in mixture, an inhibition of the bacterial growth was observed. It is important to note that at low concentrations, the studied metals are used as micronutrients for cellular metabolism [43,44], which corroborates the results found by Sengor and Gikas [42]. However, at high concentrations, they become toxic, even separately, as pointed out by Moberly et al. [45]. These researchers performed toxicity studies using *Arthrobacter* sp. and found that when Zn was present at concentrations above 10 mg/L, the bacterial growth was totally inhibited. The toxic effects of heavy metals on microorganisms may result, according to Nies [46], from the displacement and/or replacement of essential cell ions and the blocking of functional groups of enzymes, poly nucleotides and essential nutrient transport systems. Despite the toxic effect of heavy metals on bacterial growth, several species of *Arthrobacter* are commonly isolated from contaminated areas [45,47,48], which demonstrates a long-term adaptability of these organisms to metals.

The ability of *R. viscosum* to adapt to contaminants was evaluated in the presence of atrazine, Zn and Cu. The concentration of biomass obtained after 24 h of exposure to a ternary mixture of atrazine/Zn/Cu with different initial concentrations was measured and compared to that obtained for a culture previously adapted to these contaminants (according to methodology described in Section 2.3). A control assay without any contaminants in the culture medium was performed for comparison purposes, which was established to be 100% of growth (Figure 4).

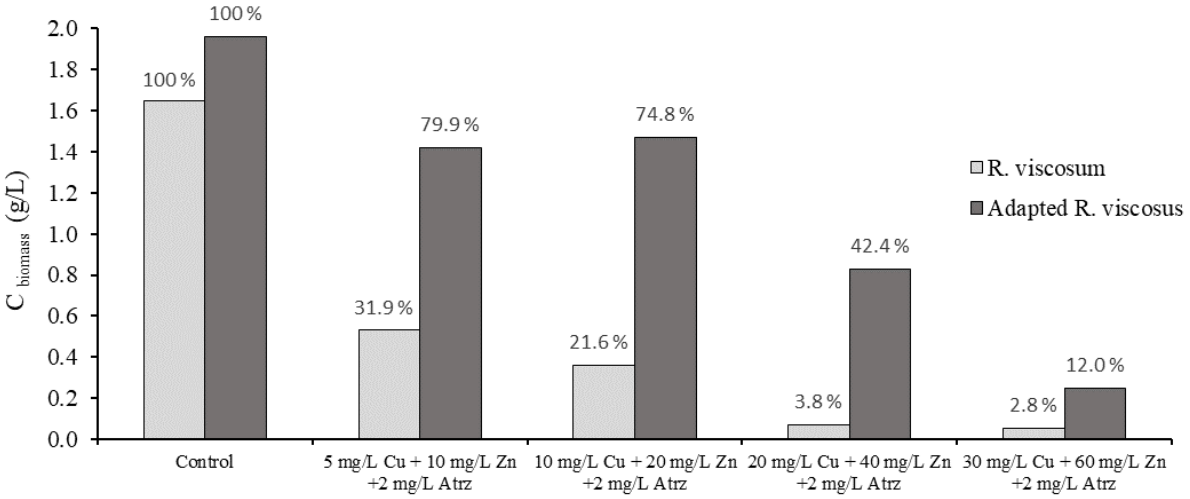

Figure 4. Growth of a pure culture of *R. viscosum* (adapted and not adapted) after 24 h of exposure to contaminants (atrazine, copper and zinc).

According to the results presented in Figure 4, it is clear that the hypothesis of bacteria adaptation is acceptable, since the difference between the concentration values of the biomass obtained without adaptation and those of the biomass previously acclimated with atrazine, Cu and Zn for the different tested cocktails of these xenobiotics is evident. These results can be attributed to the ability of bacteria to develop detoxification strategies when successively exposed to environments contaminated with heavy metals [47]. The detoxification mechanisms developed by bacteria can be classified into: enzymatic detoxification, exclusion by permeability barrier, active transport efflux pumps, intra and extracellular sequestration [49]. Extracellular sequestration is possible due to the production of extracellular polymeric substances (EPS), compounds capable of binding metal ions as a result of electrostatic interactions and, consequently, capable of keeping them out of the cell [50].

Thus, the study of the adaptation of bacteria indicated another pathway for the biosorption assays, justifying the study of the capacity of metals and atrazine sequestration by comparing non-adapted and previously acclimated bacteria.

3.3. Biodegradation of Atrazine

Biodegradation experiments were performed to assess the ability of *R. viscosum* to biodegrade atrazine, during which, atrazine was the sole source of nitrogen and carbon available for bacterial growth. Figure 5 shows the concentration of biomass over time for the biodegradation experiments performed at different initial concentrations of atrazine.

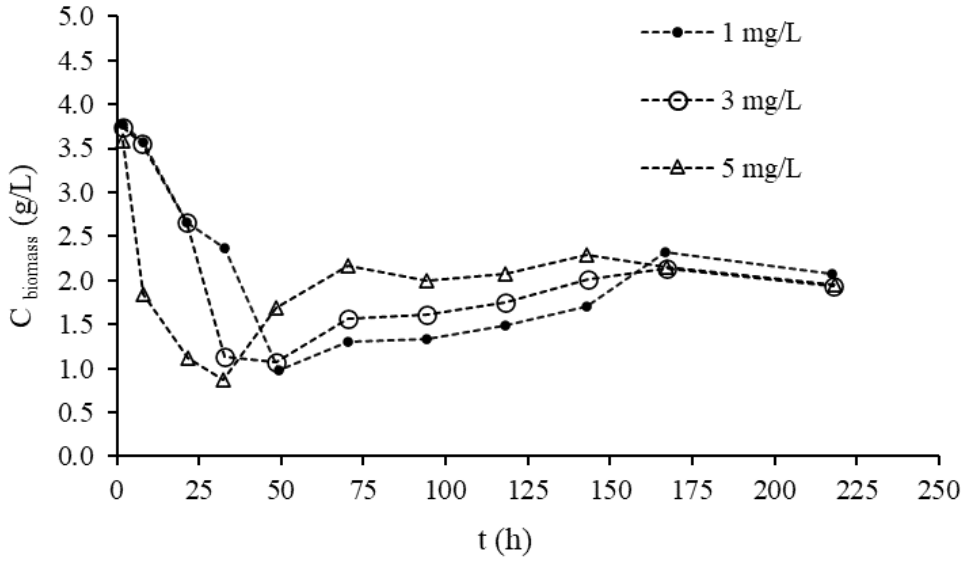

Figure 5. Concentration of *R. viscosum* biomass as a function of time for the biodegradation experiments.

It can be observed that during the first hours of the assays, the initial biomass concentration undergoes a significant decrease. This can be explained by the initial toxic effect of atrazine, since the culture was initially grown in an uncontaminated medium, being exposed to the chemical compound already in its mature phase when all the enzymes necessary for cell growth are already formed. However, after 48 h, biomass growth was observed for all atrazine concentrations, which demonstrates the adaptation of the bacteria to the contaminated environment. These results are in agreement with the results presented by other authors who evaluated the behavior and quantified the microbial growth in successive exposures to atrazine, in soil and in liquid media. Fang et al. [37] measured the average well color development (AWCD) of the soil and used this value as an indicator of the overall microbial activity. These authors found that the soil microbial functional diversity presented a trend of "suppression–recovery–stimulation," which was related to the increased degradation rate of atrazine.

Regarding the removal of atrazine, it can be found that the complete removal was not attained for all concentrations tested. For the lowest concentration of atrazine, 1 mg/L, there was a reduction of 38% of the herbicide in solution, while for the higher concentration, 5 mg/L, a removal of 32% was attained. The removal rates observed may be attributed to two distinct processes: adsorption performed by the functional groups present on the biomass surface and/or biodegradation through metabolic processes capable to degrade atrazine. In the present study, it was not possible to detect or identify any metabolite resulting from the degradation of atrazine. Therefore, the removal rates attained in this work can be attributed to either passive uptake and/or biodegradation. A schematic representation of the possible metabolic and nonmetabolic pathways for the biosorption or biodegradation of organic contaminants was previously published by Costa et al. [51], which is reprinted with permission in Figure 6.

Several studies found in the literature cite different strains of the genus *Arthrobacter* as capable of degrading atrazine at very high rates [39–41]. However, these studies are usually performed with strains that have been isolated from historically herbicide-contaminated soils, i.e., with microorganisms that have already developed specific metabolic mechanisms for contaminated environments. Udiković-Kolić et al. [52] reported the development of a

hydrolase gene in some soil bacteria after a long period of exposure to atrazine, which was capable of catalyzing the degradation process. In addition, some studies reveal that with successive exposures to higher concentrations of contaminants, microorganisms become increasingly capable of using these compounds as a source of nitrogen, carbon and energy, with the rate of degradation being increased over time. Similarly, Zablotowicz et al. [53] reported that the degree of atrazine mineralization performed by indigenous soil microorganisms increased from 10% to 60%, between the 30th day of the first application and the 7th day of the second application. These authors concluded that repeated exposure to the herbicide led to higher rates of degradation.

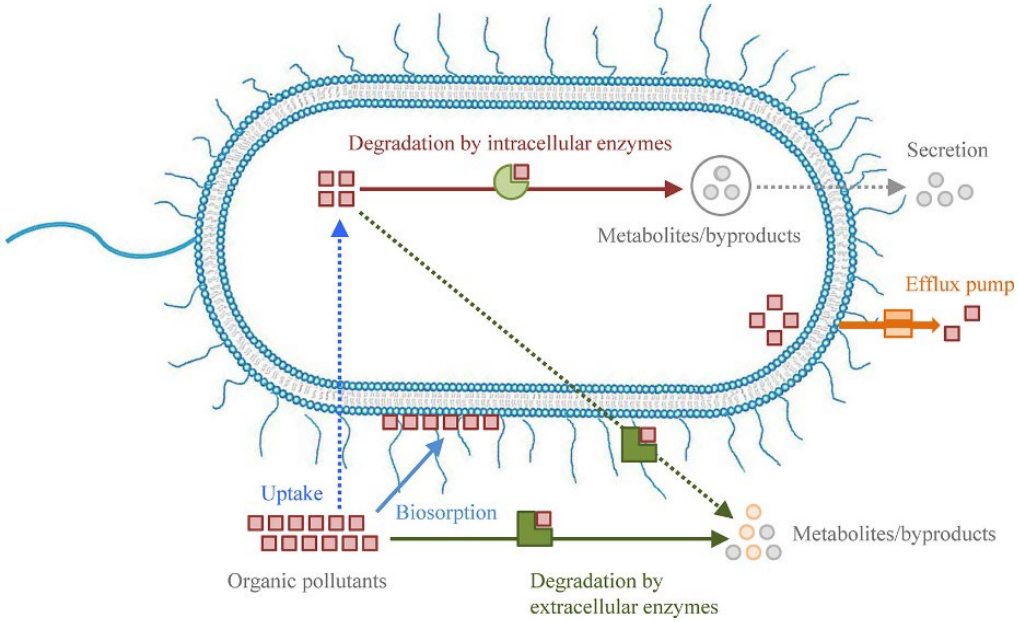

Figure 6. Possible biological interactions between cells and organic pollutants. Reprinted (adapted) with permission from [51]. Copyright (2019) American Chemical Society.

3.4. Biosorption of Atrazine and Heavy Metals

Table 1 presents the removal percentages of atrazine, Cu and Zn obtained in the experiments conducted under different types of sorption systems: zeolite, *R. viscosum* supported on zeolite and adapted *R. viscosum* supported zeolite.

Table 1. Removal percentages of atrazine (1 mg/L), Cu and Zn (40 mg/L) for the different experiments performed.

	Removal (%)		
	Zeolite + Bacteria	**Zeolite + Adapted Bacteria**	**Zeolite**
Atrazine	53.3 ± 5.3	47.3 ± 1.8	35.3 ± 1.9
Cu	59.3 ± 2.4	61.5 ± 3.4	73.2 ± 2.9
Zn	74.6 ± 3.7	81.4 ± 3.2	76.7 ± 2.3

These results show higher removal percentages for the sorption systems with supported bacterial biofilm in comparison to the ones only with zeolite. Such results suggest that the improvement in atrazine removal is attributed to the role of bacteria, which can perform passive uptake (biosorption) and/or biodegradation. The results obtained for the systems with or without previous adaptation of the bacterium to the contaminants were very similar, although a slightly better performance was noticed for the system without previous adaptation, which attained a maximum removal rate of 53.3%. None of the studied sorption systems were able to completely remove atrazine, which can be due to the low

extension of the adsorption process. The adsorption of atrazine depends on its degree of protonation, which is itself a function of its pKa value and of the pH of the solution. With a pKa value of only 1.68, atrazine is considered a weak basic herbicide. Therefore, at the pH value used in the biosorption experiments (starting pH 4.7), the non-protonated molecules dominate over the protonated species. Thus, the electrostatic interaction between the molecules and the negatively charged surface of the zeolite is not favored and the retention of the molecules on the surface only takes place through weak forces such as hydrogen bonds or Van der Waals forces [54,55]. Accordingly, Lemić et al. [56] reported a lower adsorption capacity for atrazine in comparison to other pesticides, when organo-zeolites were used as adsorbent material. The maximum uptake of atrazine was found to be 2.01 μmol/kg, while higher adsorption capacities were reported for lindane and diazinone, 3.40 and 4.42 μmol/kg, respectively. The adsorption of atrazine was reported to be enhanced after acid activation of the zeolite surface. Salvestrini et al. [57] performed adsorption experiments with atrazine (1–25 mg/L), comparing the adsorption capacity of zeolite-rich tuffs before and after acid activation. These authors reported that the maximum uptake increased from 0.55 mg/g to 1.1 mg/g after the acid activation of the zeolites, for an initial concentration of atrazine of 25 mg/L. In this work, the uptake of atrazine obtained for the experiment with zeolite (without bacteria) was 0.019 mg/g, which is a lower uptake in comparison to the uptake values obtained in the work of Salvestrini et al. [57]. However, it is important to note that these authors used a ratio of 2.5 mg of atrazine per gram of zeolite (80 mg of zeolite with 8 mL an atrazine solution of 25 mg/g), while in the present study, a significantly lower ratio, 0.03 mg of atrazine per gram of zeolite, was used. Since these mass ratios correspond to the maximum uptake values that theoretically can be achieved, in the present work, about 63% of the maximum adsorption capacity of the zeolite was attined, while the zeolite-rich tuffs studied by Salvestrini et al. [57] reached only 44% of the maximum uptake value, even after the acid activation pre-treatment. Furthermore, in the present work, the uptake value attained for atrazine may be influenced by the concomitant presence of metal ions in solution.

As can be observed in Table 1, the removal percentages of Cu and Zn were not significantly different when comparing the zeolite and the zeolite with bacterial biofilm. Thus, the role of the bacterium on the overall removal of Cu and Zn was not evident in the performed studies, probably because the adsorption performed by the zeolite was the dominant process for metals removal. In fact, the biomass used in the biosorption experiments were in much lower concentration in comparison to the zeolite concentration. The biosorption experiments were performed using 5 g of zeolite and 15 mL of *R. viscosum* culture with an initial biomass concentration of about 1.5 g/L (which gives approximately 0.23 g of biomass). Thus, a mass ratio of almost 22 g of zeolite per gram of bacteria was used, which can be an explanation for the residual role of the bacteria on the overall removal on the biosorption of Cu and Zn.

Comparing the removal of Cu and Zn for all the sorption systems under study, higher removal percentages were obtained for Zn in comparison to Cu. These findings are not in agreement with several works found in the literature, which report that zeolites have higher selectivity to Cu in comparison to Zn [58–60]. Erdem et al. [61] studied the removal of heavy metals by natural zeolites and concluded that the adsorption process relies on bulk density and size of hydrated ion, with the following selectivity order being observed: $Co^{2+} > Cu^{2+} > Zn^{2+} > Mn^{2+}$. Since pesticides have different coordination ability with metal ions, the presence of atrazine possibly interfered with the adsorption of Cu and Zn, shifting the theoretical selectivity order for the adsorption on zeolites. Theoretical studies performed by Meng and Carper [62] show that atrazine can form complexes consisting of one or two atrazine molecules with one metal ion including variable water and chloride ions. Meng and Carper [62] reported that Cu forms 1:2 complexes while Zn forms 1:1 complexes. The results obtained in the present work suggest that Zn-atrazine complexes have higher affinity for the adsorption on the zeolite surface than Cu-atrazine complexes,

which can be related with steric factors and the higher stability of Zn-atrazine complexes over Cu-atrazine complexes.

3.5. Mathematical Modeling of Biosorption Data

The pseudo-first and pseudo-second order models were used to fit the kinetic data. The results of mathematical modeling for the biosorption of atrazine and heavy metals obtained by the application of the pseudo-first and pseudo-second order models are presented in Tables 2 and 3, respectively, with the calculated parameters, correlation coefficient (R^2), kinetic constants (k_1 and k_2) and maximum amount adsorbed (q_e).

Table 2. Pseudo-first order model fittings for the biosorption/adsorption of atrazine, Cu and Zn for the different sorption systems in study.

	Zeolite + Bacteria			**Zeolite + Adapted Bacteria**			**Zeolite**		
	k_1 (h^{-1})	**q_e (mg/g)**	**R^2**	**k_1 (h^{-1})**	**q_e (mg/g)**	**R^2**	**k_1 (h^{-1})**	**q_e (mg/g)**	**R^2**
Atrazine	0.068 ± 0.015	0.033 ± 0.001	0.984	0.226 ± 0.020	0.030 ± 0.004	0.999	0.132 ± 0.014	0.020 ± 0.004	0.998
Cu	0.057 ± 0.014	1.00 ± 0.053	0.975	0.077 ± 0.014	1.07 ± 0.031	0.992	0.125 ± 0.018	1.23 ± 0.013	0.999
Zn	0.221 ± 0.030	1.55 ± 0.036	0.993	0.160 ± 0.030	1.63 ± 0.012	0.999	0.112 ± 0.047	1.61 ± 0.006	0.999

Table 3. Pseudo-second order model fittings for the biosorption/adsorption of atrazine, Cu and Zn for the different sorption systems in study.

	Zeolite + Bacteria			**Zeolite + Bacteria Adapted**			**Zeolite**		
	k_2 (g/mg.h)	**q_e (mg/g)**	**R^2**	**k_2 (g/mg.h)**	**q_e (mg/g)**	**R^2**	**k_2 (g/mg.h)**	**q_e (mg/g)**	**R^2**
Atrazine	4.52 ± 2.62	0.034 ± 0.002	0.980	26.53 ± 5.98	0.030 ± 0.004	0.999	18.13 ± 6.26	0.020 ± 0.006	0.997
Cu	0.091 ± 0.037	1.09 ± 0.061	0.987	0.161 ± 0.065	1.13 ± 0.043	0.993	0.447 ± 0.138	1.26 ± 0.015	0.999
Zn	0.318 ± 0.116	1.58 ± 0.052	0.992	0.663 ± 0.226	1.65 ± 0.011	0.999	0.318 ± 0.115	1.64 ± 0.024	0.999

For all of the sorption systems under consideration, both pseudo-first order and pseudo-second order kinetic models fit experimental data with correlation coefficients (R^2) higher than 0.97. Statistical analysis indicates that both fittings are very similar with a good prediction of the experimental q_e values. It is not possible to conclude about the mechanisms involved in the biosorption/adsorption of atrazine and of heavy metals since these mechanisms are hardly assigned based on observed kinetic experiments or by fitting kinetic models [63,64].

3.6. Permeable Bio-Barrier Reactor

The removal of contaminants over time during the experiment performed on the permeable bio-barrier reactor is shown in Figure 7. A total of 30 L of solution were passed through the bio-barrier and kept in recirculation, without the addition of more contaminants.

After 120 h of operation in recirculation mode, the bio-barrier was able to remove 80% of copper and more than 95% of zinc. Similar to the results obtained during the biosorption experiments, the bio-barrier consisting of *R. viscosum* supported on zeolite was selective to Zn over Cu. Regarding the removal of the herbicide, after 3 h of the beginning of the assay, the bio-barrier was able to remove 54% of atrazine. However, the retention of atrazine decreased over time, and at the end of the experiment, only 25% of atrazine was removed from the liquid medium. The fact that the atrazine molecule is generally in its non-ionized form in solutions with pH values close to neutrality makes it difficult to be adsorbed by an inorganic adsorbent such as zeolite or to be biosorbed by the bacterial biofilm. Another explanation for the desorption of atrazine is that the retention of these molecules to the zeolite surface only takes place through weaker forces such as hydrogen bonds or Van der Waals forces [54,55], allowing their leaching.

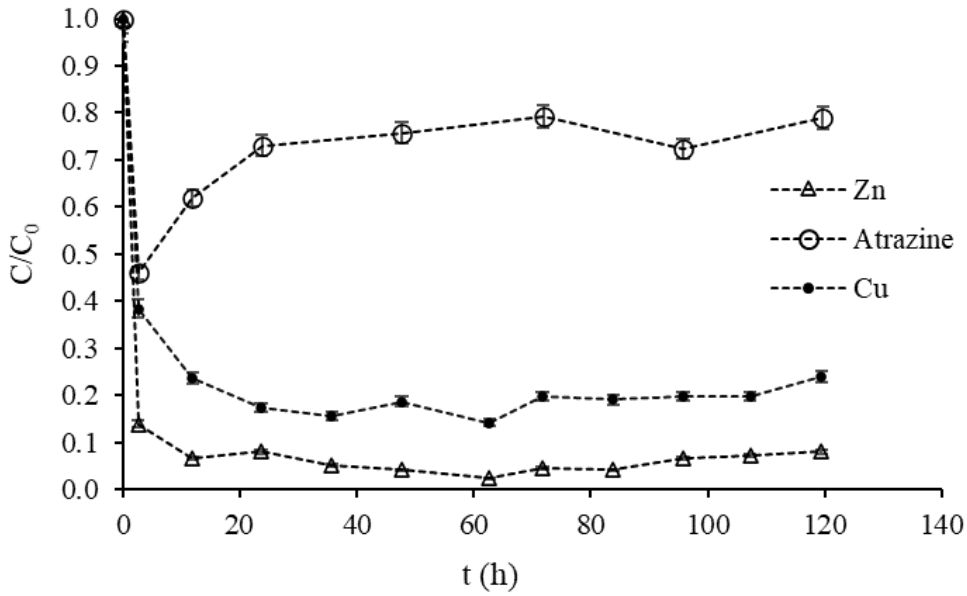

Figure 7. Removal of contaminants over time during the PBR experiment.

The preliminary results shown in this work present a new perspective that can and should be improved for the treatment of recalcitrant contaminants, such as herbicides, as well as inorganic pollutants, being a low cost and sustainable alternative to conventional treatment systems.

3.7. Characterization Procedures

FTIR spectra of the starting 13X zeolite and of atrazine are presented in Figure 8 (left). The spectrum of the starting zeolite presents the typical broad absorption band centered at 3400 cm^{-1}, which corresponds to OH vibrations due to the presence of the hydroxyl-groups from the water molecules that are physically adsorbed. The band centered at 1640 cm^{-1} could be attributed to the presence of water molecules because of the ν (H-OH-) vibration band [65]. The bands observed in the range of 1250−900 cm^{-1} and 720−650 cm^{-1} correspond to internal vibrations of the framework TO_4 tetrahedron [66].

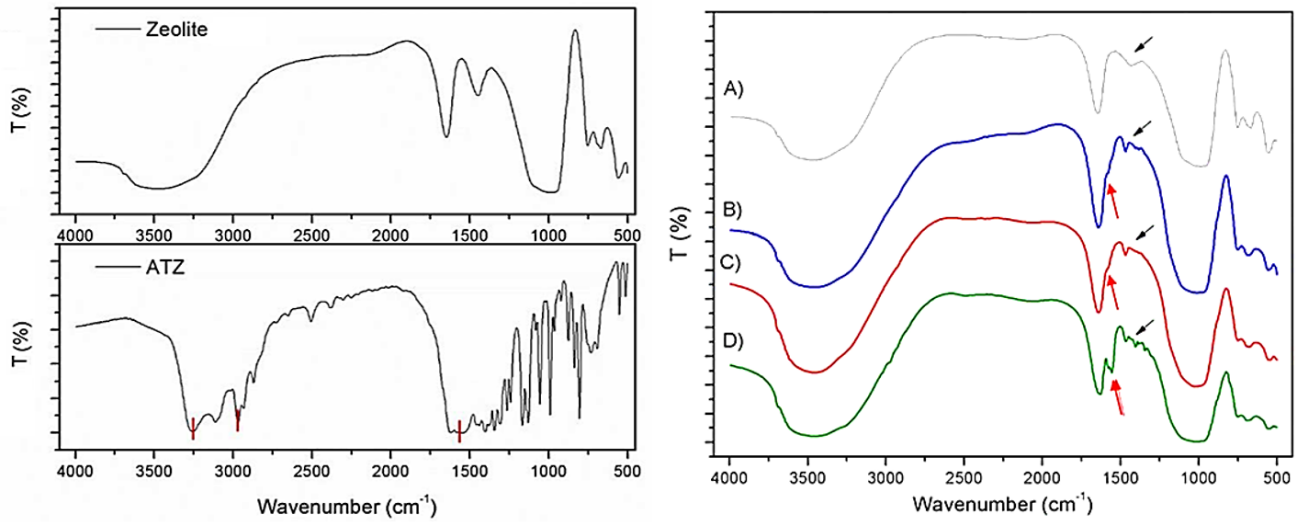

Figure 8. FTIR spectra of the starting 13X zeolite and atrazine (**left**) and of zeolite samples obtained after batch and reactor experiments (**right**): (A) permeable barrier reactor; (B) biosorption by bacteria/zeolite; (C) biosorption by adapted biofilm/zeolite; (D) adsorption with zeolite. Black and red arrows indicate bands centered at 1300 cm^{-1} and 1550 cm^{-1}, respectively.

Figure 8 (right) shows the FTIR spectra of different zeolite samples taken after the experiments: permeable barrier reactor (A), biosorption with bacteria/zeolite (B), biosorption with adapted bacteria/zeolite (C) and adsorption with zeolite (D).

For all samples (A, B, C and D), the band centered around 1300 cm^{-1}, assigned to the C-H angular deformation, suffered changes in intensity and shape, which may be associated with the interaction with metals in solution, as described by Quintelas et al. [67]. In addition, an intense peak at 1550 cm^{-1} can be observed in spectrum D (experiment performed only with zeolite), while for spectra B and C (samples of bacteria supported on zeolite), only a weak signal is observed in this region. This peak can be attributed to the N-H bending of primary amine present in atrazine molecules [68]. These observations may be an indication that atrazine can be degraded by the bacterial biofilm, which explains that the peak at 1550 cm^{-1} has a lower signal in comparison to that observed for the zeolite without bacterial biofilm. Thus, it is believed that the removal of atrazine occurs not only by biosorption (passive uptake) but also by metabolic degradation, i.e., biodegradation (active uptake).

The morphology of the starting 13X zeolite was evaluated by SEM (Figure 9). SEM images reveal a crystal-like surface morphology although with a non-uniform crystal size. The porous nature of the zeolite, along with the depressions and grooves on its surface, suggests the presence of a large number of binding sites for the fixation of inorganic and organic pollutants. The growth and development of the bacterial biofilm on the zeolite surface was also evaluated by SEM (Figure 10), whose images revealed an uniform growth of the biofilm that adhered to the grooves and cavities of the zeolites and, thus, a well-established biofilm.

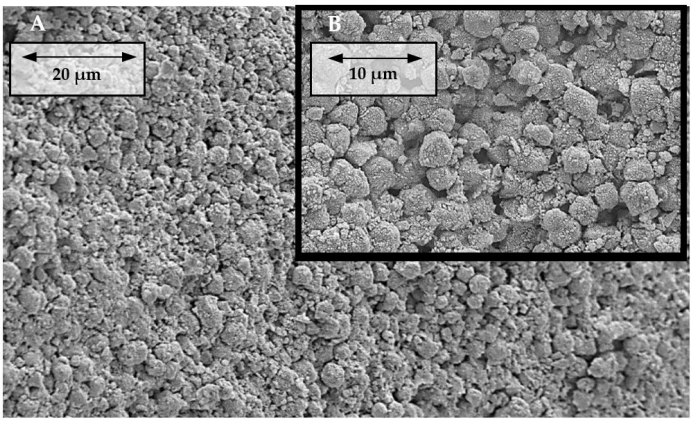

Figure 9. SEM image of the starting 13X zeolite: magnifications of 1000× (**A**) and 3000× (**B**).

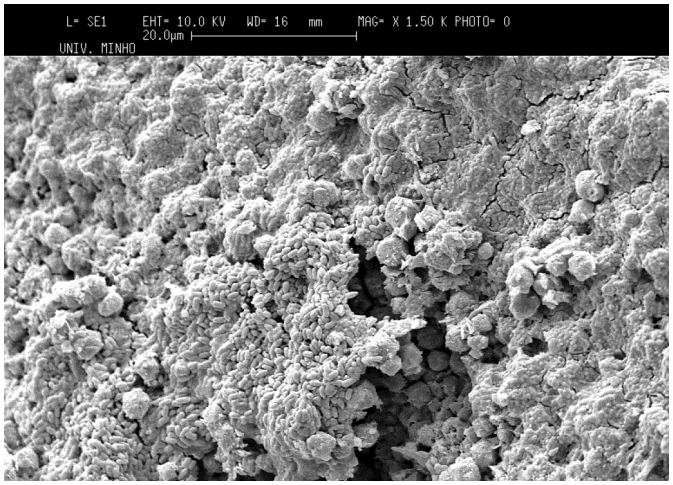

Figure 10. SEM image of *R. viscosum* biofilm supported on 13X zeolite (magnification of 1500×).

4. Conclusions

This work focused on the development of a permeable bio-barrier consisting of a bacterial biofilm supported on zeolite to be applied for the removal of atrazine associated with copper and zinc from liquid media.

The toxicity experiments reveal that Cu and Zn have a xenobiotic effect over *R. viscosum*, even at low concentrations (5 mg/L). However, the growth of *R. viscosum* was not affected by high concentrations of atrazine, up to 7 mg/L. The results obtained demonstrate the ability of *R. viscosum* to interact and to adapt to the contaminated environment, since the pre-acclimation of the bacteria to the contaminants allowed for an increase of 50% of the bacterial growth. This feature favors its application in remediation technologies. The role of *R. viscosum* on the overall removal of Cu and Zn was not significant, since the adsorption performed by the zeolite was the dominant process for metals removal. Regarding the herbicide, two distinct processes, adsorption and biodegradation through metabolic processes, were proposed to be involved in the removal of atrazine. The development of a permeable bio-barrier with immobilized biofilm was successfully performed, with removal rates of 85% for copper, 95% for zinc and 25% for atrazine, showing the potential of this system for its application in sustainable and low-cost bioremediation systems.

The development of permeable bio-barrier treatment systems is a challenging issue, and deeper studies that simulate more realistic scenarios are needed, aiming for the successful implementation of the technology.

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Conflicts of Interest: The authors declare no conflict of interest.

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