**Novel magnetic torus microreactors for the removal of phenols and dyes aided by laccase immobilized on magnetite nanoparticles**

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# Abstract

In this work, the design, manufacture and testing of three different magnetic microreactors based on torus geometries (i.e., one-loop, two-horizontal-loop, and two-vertical-loop) is explored to increase the enzyme-based degradation of dyes by laccase bionanocomposites, improve the particle suspension and promote the interaction of reagents. The laccase enzyme was covalently immobilized on amino-terminated silanized magnetite nanoparticles (Lac-Magnetite) and the impact of varying the pH on the catalytic performance of the obtained bionanocomposites was analyzed. The optimal configuration for the torus microreactor and the applied magnetic field was evaluated in silico with the aid of the CFD and particle tracing modules of Comsol Multiphysics®. The Eriochrome Black T (EBt) dye was tested as a biodegradation model at three different concentrations 5 mg/L, 10 mg/L and 20 mg/L. The phenol oxidation/removal was evaluated on artificial wastewater and real wastewater. The optimal performance of the bionanocompound was achieved in the range of pH 4 to 4.5. A parabolic movement on the particles was induced by the magnetic field, which led to breaking the stability of the laminar flow and improving the mixing processes. Based on the simulation and experiments conducted with the three geometries, the two-vertical-loop demonstrated a better performance mainly due to larger dead zones and longer residence time. Also, the overall dye removal efficiencies for this microreactor and the Lac-Magnetite bionanocompound were 98.05%, 93.87%, and 92.74% for the three concentrations evaluated. The maximum phenol oxidation with the Lac-Magnetite treatment at low concentration for the artificial wastewater was 79.89% while its phenol removal efficiency for a large volume of real wastewater was 17.86%. This is equivalent to 200 working biodegradation cycles for the dyes.

**Keywords**: phenol, removal, bionanocomposites, laccase, magnetic nanoparticles, microreactor, torus.

# Introduction

Water pollution is one of the main environmental problems that mankind is facing over the coming few decades [1][2][3]. Oceans, aquifers, rivers, lakes, and groundwater are being polluted at an unprecedented rate [2][4][5][6]. This directly affects not only the health of an increasingly higher number of human communities but ecosystems of other living organisms [3]. Industrial and agricultural processes are thought to be responsible due to the continuous discharge of large volumes of wastewaters to these aquatic environments [7][8]. Some of the most common discharged pollutants include pathogens, excess nutrients, suspended solids and sediments, pesticides, plastics, fertilizers, acids, detergents, pharmaceuticals, phenols, minerals, dyes and pigments, and heavy metals [9][10].

The annual industrial production of synthetic or azo dyes approaches 70 million tons [11]. These xenobiotic chemicals are not normally encountered in nature but show superior solubility in water and consequently, stand as one of the major sources of water pollution [12][13][14]. Due to their ease of production, azo dyes are ubiquitously found around the world in a number of industries including textiles, leather goods, paper, plastics, foodstuffs, cosmetics and candles [15][16]. Azo dyes are used to develop vivid colors, most notably yellows, oranges and reds [16]. In recent years, the growing demand for these products has led to an increased production of dyes and as a result, the amount of waste produced [14].

Despite the industry's efforts to couple wastewater treatment processes to their continuous production process, 90% of reactive textile dyes entering activated sludge sewage treatment plants will pass through unchanged [17][13]. As a result, between 30 to 150 thousand tons of dyes are discharged into water bodies, soil and aquatic ecosystems annually [18][11].

The continuous exposure to dyes is harmful to life in general [11]. Water sources contaminated by these compounds have low penetration of sunlight and oxygen, which limits the survival of various aquatic forms [18]. Additionally, it promotes the anaerobic degradation, which entails the formation of sub-products of high biological toxicity that might ultimately end up in the food chain. Furthermore, many dyes are made from known carcinogens and toxins, such as benzidine and other aromatic compounds, whose main effects to humans are related to irreversible damage to DNA and proteins [13][18][16].

Current regulations in Europe, China, Taiwan, Korea and Japan limit the release of these synthetic dyes to approximately 30 parts per million when used in the production of textiles and leather items intended for prolonged contact with the skin [16]. This more stringent regulatory framework is forcing industry to find methods to lower the color content in their wastewater effluents before their final discharge into surface waters [16][18].

The primary methods to remediate azo compounds are coagulation/flocculation, adsorption, precipitation, flotation, membrane filtration, chlorine disinfection bioflocculants, ion pair extraction, ultrasonic mineralization, electrolysis, ion exchange, advanced oxidation processes, sonication, photocatalysis, and ozonation [3][11][12][15][18]. Some of these processes; however, are difficult to operate, rely on costly feedstocks, require complex instrumentation and control schemes, exhibit limited versatility, and are highly interfered by other wastewater pollutants. Moreover, they can generate genotoxic or hazardous byproducts [15][19][20]. This has led to the need of considerable investments for their full implementation or the development of advanced water treatment technologies [8][21]. One attractive alternative to overcome some of these issues is the incorporation of enzyme-based biocatalysts as active components into remediation processes. This approach facilitates the degradation of the recalcitrant organic compounds, including the azo dyes, without introducing any extra toxic components, the processing of an ample range of pollutants concentrations, require shorter treatment times, and involve low operation costs [22][23][24]. This has attracted significant attention over the past few years, and consequently a number of contributions have emerged describing the considerable potential of the biocatalytic degradation of azo dyes and wastewater treatment [14][17][12]. One of the most attractive family of enzymes for bioremediation of azo dyes are laccases [25]. These enzymes are oxidoreductases capable of oxidizing phenolic compounds into phenoxyl radicals, with the aid of 4 copper electrons in their structures [14][12][23]. The presence of aromatic polyphenolic components in azo dyes make them suitable targets for decolorization with laccases [12][23].

Nonetheless, there are some shortcomings for bioremediation processes enabled by free enzymes, which might limit their scaling-up and eventual industrial implementation. These issues include their costly and relatively tedious isolation and purification, their short lifetimes, and the decrease in their catalytic activity and stability due to the harsh physicochemical conditions normally found in industrial effluents [23][26]. An avenue to overcome these issues is the immobilization of enzymes as it has demonstrated to increase their enzymatic activity, long-term stability as well as to facilitate their possible recovery and reuse. The principal immobilization methods for Laccases are adsorption, self-immobilization, covalent binding, mesh embedding, microencapsulated embedding, and two-step combination [27]. Moreover, recent developments have advanced in enabling their incorporation into continuous treatment processes, which might be beneficial for low-cost industrial applications [23][28][29].

Microreactor devices have been recently developed by taking advantage of important advances in the microfluidics field. In particular, the ability to precisely control and manipulate micro- and nano-scale objects transported within precisely designed and manufactured microchannels with unique 2D and 3D geometries [30]. One of the most attractive features of these devices is that they can carry out chemical processes with low reagent consumption due to the small sample volumes handled. There are different device configurations and peripherals that have been developed to assemble systems capable of complying with different analysis and functions including sampling, sample processing and in-line real-time monitoring, and processing of the collected data. With the advent of easier and cheaper ways of manufacturing at the microscale, the field of microfluidics has had an exponential growth and therefore has reached a next level of maturity [31]. In consequence, new perspectives have emerged regarding the possibilities for cost-effective, large-scale implementation in several industries including microelectronics, pharma, food, health and cosmetics [31][32]. This rapid prototyping approach has been complemented with powerful *in silico* tools that significantly accelerate the performance analysis of novel devices [33]. One of such tools is CFD simulations of the fluid flow and transport of objects within the microsystems. In this approach, the channel geometries become the computational domains that are discretized to numerically solve the momentum transfer equations coupled to supporting equations describing the movement of the objects. With the simulation results, manufacturing takes shorter times and favor only prototypes with the highest performance, which can be further optimized with much less investment [34][35].

Here, we explore the design and manufacture of microreactors with toroidal topologies to enable the enzyme-based degradation of dyes. We hypothesize that such microreactors are suitable for maximizing biodegradation processes due to the absence of dead volume, the efficient mixture of reagents, and the possibility of continuous reaction within the toroidal loop [36]. A first attempt to find an optimal configuration for the torus microreactor was explored *in silico* with the aid of Comsol Multiphysics® by analyzing mixing patterns and fluid dynamics. This was accomplished by coupling the CFD and particle tracing modules. Low-cost device prototyping was conducted in polymethyl methacrylate using a laser cutting system and commercially available fittings for the assembly and subsequent testing. The microreactor´s potential for biodegradation was evaluated for laccases covalently immobilized on amine-terminated silanized magnetite (Fe3O4). The model reaction was the degradation of the commercially available dye Eriochrome Black T (EBt). To maintain the nanoparticles suspended during the treatment process and maximize contact between the components; one or two permanent magnets were coupled to the microreactor. Finally, the extent degradation of the azo molecules was examined in a real wastewater sample by estimating quantitative environmental parameters of water quality and the removal of phenolic compounds.

# Materials and Methods

## Materials

Polymethyl methacrylate (PMMA), Methyl methacrylate, Ethanol (96%) and 345 mT Neodymium cylindrical magnets (ϕ: 6 mm x h: 7 mm) were purchased at a local store. 2,2-azino-bis(3-ethylbenzothiazoline-6) sulphonic acid (ABTS), glutaraldehyde (25%), sodium hydroxide (NaOH) (98%), tetramethylammonium hydroxide (TMAH) (25%), 3-Aminopropyl-triethoxysilane (APTES) (98%) were purchased from Sigma-Aldrich (USA). Iron (II) chloride tetrahydrate (98%) (FeCl2\*4H2O), Iron (III) chloride hexahydrate (97%) (FeCl3\*6H2O), phenol crystallized (99,5%) (C6H6O) (Phenol) and dye Eriochrome Black T (EBt) (C.I. 14645) were obtained from PanReac AppliChem (Spain).

## Laccase

Laccases from *Pycnoporus Sanguineus* CS43 (EC 1.10.3.2) were obtained from tomato medium as described elsewhere [37]. In brief, mycelia were removed from the culture supernatant by filtration using two tangential flow filters in series, with pore sizes of 0.5 mm and 0.2 mm, respectively. The obtained laccase cocktail was ultra-filtered using a membrane with a molecular weight cut-off of 10 kDa.

## Synthesis of magnetite nanoparticles

Magnetite nanoparticles were synthesized via coprecipitation by mixing 20 mL of 1M FeCl2 and 20 mL 2M FeCl3 under agitation at 1,500 RPM and 90°C. Subsequently, 40 mL of 8M NaOH and 40 mL 2% (v/v) of TMAH were added to the mixture during 3.5 h at a flow rate of 12 mL/h. The obtained magnetite nanoparticles were purified by magnetic separation with the aid of a strong permanent magnet, then washed thoroughly with 2% (v/v) TMAH, and finally sonicated for 100 min using a VibraCell sonication system (Sonics, USA). The synthesized magnetite nanoparticles exhibited an average size distribution of 88.59 𝑛𝑚 with a polydispersity index of 0.182 as determined by DLS analysis with the aid of a Zetasizer Nano ZS, (Malvern, USA). The nanoparticles morphology was analyzed as previously described by Lopez-Barbosa *et al*. (2020) [38].

## Enzyme immobilization and pH stability

### Enzyme immobilization on magnetite nanoparticles

Nanoparticles were buffered using a NaOH solution 1M until reaching pH 11, then sonicated for 10 min. Next, 50 µL of 2% (v/v) TMAH was pipetted and the resulting mixture sonicated for 10 min. Silanization was carried out by adding 50 µL of 2% (v/v) APTES and then, the mixture was sonicated again for 20 min. Subsequently, 50 µL of 2% (v/v) glutaraldehyde was added to the mixture as the crosslinker, and left to react for 30 min. Finally, 50 µL of 960 U/L laccase enzyme was added and left overnight to immobilize on the surface of the nanoparticles to achieve the covalent bonding. The resulting bionanocomposites (Lac-Magnetite) were separated with the aid of a strong permanent magnet and thoroughly washed with Milli Q water.

### pH impact on the enzymatic activity

Stability of free and immobilized laccase (Lac-Magnetite) under different pH values was examined with the aid of phosphate–citrate buffer solutions, at pH values of 2.0, 3.0, 4.0, 4.5, 5.0, 6.0, 7.0 and 10.0, at 25°C. Laccase activity, of free and immobilized laccase, was determined spectrophotometrically as described by Moilanen *et al*. (2010) [21] at 436 nm using a GENESYS 10S UV-Vis v4.004 2L5R078128 (Thermo SCIENTIFIC, USA), . One activity unit was defined as the amount of enzyme that oxidized 1 µmol of ABTS per min. The activities were expressed in U/L. All measurements were carried out in triplicate.

## Microreactors design and manufacture

### Microreactor geometry design and simulation

Three different geometries were studied *in silico* via Comsol Multiphysics 5.3® for the torus microreactor. Figure 1 shows such geometries, namely, one-loop, two-horizontal-loop, and two-vertical-loop microreactors. For each microreactor, the Computational Fluid Dynamics (CFD) and Magnetic Field, no current (MF) modules of Comsol® were coupled to simultaneously simulate the hydrodynamics and impact of the imposed magnetic fields, respectively. To evaluate the impact of magnetic fields on the transport of the individual nanoparticles, the Particle Tracing (PT) module was implemented solely for the one-loop microreactor.

C:\Users\MULTINSA\Desktop\Imagenes paper juli cambiadas\F1 simulation and diagram.tif

**Figure 1.** Simulation geometries and manufactured microreactors. A) One-loop, B) Two-horizontal-loop and C) Two-vertical-loop. D) Experimental diagram for the biodegradation process. The numbers 1, 2, 3, 4 and 5 in the figure correspond to the solution injection, microreactor, permanent magnet, treated sample and absorbance analysis, respectively.

The laminar flow module (Eq. (1)) was used here to describe the fluid flow due to the low Reynolds number (Re) calculated for the microreactors (Re = 4.4). The inflow to the microreactor was 12 mL/h while the output pressure was set to 1 atmosphere. Density and viscosity of water were assumed as the properties of the flowing fluid. The non-slip boundary condition was imposed at the walls of the microchannels. The boundary conditions are summarized in **Figure 1**. MF was used to simulate the magnetic field generated by a neodymium permanent magnet (Eq. (2) and Eq. (3)). An additional domain representing the surrounding air was added to the computational domain. Air and water were assumed with magnetic permeability of 1. These two physics were solved simultaneously in a stationary study with direct solver PARDISO:

|  |  |
| --- | --- |
|  | Eq. (1) |
|  | Eq. (2) |
|  | Eq. (3) |

Where, is the pressure gradient in the fluid, µ is the viscosity of the fluid and is the velocity of the fluid. For the equations of MF, is a magnetic flux density, is the magnetic permeability of the vacuum, is the magnetic permeability of the fluid, is the magnetic field and is the remanent flux density of the neodymium magnet. The PT module was used to study the dynamics of the magnetic particles within the microreactor. The particle diameter was assumed as 1.2 µm with a density of 5180 kg/m3. The particle size was decided according to our own previous experimentation that demonstrated the presence of small agglomerations of NPs forming clusters (data not shown). Additionally, the magnetic permeability of the particle was set to 5000 H/m. The particles were delivered at the main inlet of the device. The PT was solved in a time-dependent study for 10 seconds using 0.1s steps. In this case, a projected conjugate gradient iterative solver was chosen due to the high demand for computational resources. Also, a parametric analysis was performed to evaluate the impact of the remanent flux density of the neodymium magnet on the particles transport. Equations Eq. (4) to Eq. (7) describe the forces experienced by a particle. This study considered the interactions between the particles and the fluid (drag force) and the magnetic attraction between particles. Particle-particle interactions, lifting force and Brownian motion were disregarded to avoid dealing with unnecessary complexity that provides little extra information at the expense of exceedingly large computational resources:

|  |  |
| --- | --- |
|  | Eq. (4) |
|  | Eq. (5) |
|  | Eq. (6) |
|  | Eq. (7) |

Where is the drag force, is the mass of the particle, is the velocity of the particle, *V* is the fluid velocity, is the density of the particle and is the particle diameter. is the magnetophoretic force, is the particle radius and is the magnetic permeability of the particle. As described before, the meshing was subjected to a convergence analysis to determine the minimum number of elements necessary to arrive to a meaningful solution. For this study, five random measurement points in the microchannel were selected along the computational domain and the change in magnitude of speed was evaluated as the number of mesh elements increased. As a convergence criterion, it was determined that the velocity magnitude change obtained for a meshing level and the next one was below 3%. An unstructured mesh with tetrahedral elements was generated. A stationary study was run with the direct solver PARDISO that allows to parallelize processes solving large symmetric or structurally symmetric dispersed linear systems of equations in shared memory multiprocessors [39].

### Manufacture of the torus microreactors

Device prototyping was conducted by engraving the microchannels (1 mm of depth) on PMMA slides of 2.5 mm thickness with an area of 75 mm x 25 mm. The proposed torus geometries were cut with the aid of a laser cutter system Speedy 100, 60 W (TROTEC, Germany). Proper assembly and sealing were achieved by gluing the slides with ethanol followed by heating at 105°C for 8 min. The device was then maintained under constant pressure with the aid of a home-made press. Inlets and outlets were equipped with commercially available fittings to facilitate connection to pumping devices (e.g., syringe pumps) (See Figure 1).

## Biodegradation studies

### Experimental tests for biodegradation of dyes

EBt dye was tested as model compound to estimate enzymatic activity at pH 5.48 and three different concentrations, namely, 5 mg/L, 10 mg/L and 20 mg/L. Each microreactor was infused with 5 mL of each dye solution at a constant rate of 12 mL/h for 25 minutes. Prior to this, 5 mg of Magnetite or Lac-Magnetite was introduced to each microreactor in the presence of the Neodymium permanent magnets, of 349.23 mT, to retain the bionanocomposites at the reaction loops. Samples were analyzed using a spectrophotometer GENESYS 10S UV-Vis v4.004 2L5R078128 (Thermo SCIENTIFIC, USA) by measuring the absorbance peak at 545 nm, which is the maximum absorbance for EBt, and the absorbance area of the visible spectrum in the range between 400 and 700 nm (See **Figure 1**). All measurements were carried out in triplicate.

The dye biodegradation was calculated as a percentage of removal according to Eq. (8). Where the is the average absorbance (at the absorbance peak or the area under the absorbance curve, respectively) of the dye at each concentration previous to entering the microreactor, and the is the absorbance of each replica.

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| --- | --- |
|  | Eq. (8) |

### Experimental tests for remediation of artificial wastewater

Artificial Wastewater (AW) was prepared at pH 4.42 and three concentrations of Phenol, namely, 5 mg/L, 10 mg/L, and 20 mg/L. Each microreactor was infused with 5 mL of each artificial wastewater solution at a constant rate of 12 mL/h for 25 minutes. Prior to this, 5 mg of Lac-Magnetite was introduced to each microreactor in the presence of the Neodymium permanent magnets to retain the bionanocomposites at the reaction loops. Samples were analyzed using a spectrophotometer GENESYS 10S UV-Vis v4.004 2L5R078128 (Thermo SCIENTIFIC, USA) by measuring the absorbance peak at 270 nm, which is the maximum absorbance for Phenol, and the absorbance area in the range between 190 and 1100 nm. All measurements were carried out in triplicate.

The Phenol oxidation was calculated as a percentage of change in the phenol composition, according to Eq. (9). Where the is the average absorbance (at the absorbance peak or area under the absorbance curve, respectively) of the phenol at each concentration previous to entering the microreactor, and the is the absorbance of each replica, while is the absorbance of the total oxidized phenol. A value of zero in the percentage corresponds to a situation of no noticeable change in the phenol contents while negative values represent a reduction in such a concentration mainly due to physical phenomena (*e.g.,* adsorption). Also, positive values might be explained by the polymerization of phenol upon oxidation by the Laccase.

|  |  |
| --- | --- |
|  | Eq. (9) |

### Treatment of a large volume of real wastewater

Samples of Real Wastewater (RW) were collected from laboratories of the School of medicine at los Andes University (Bogota, Colombia). RW was then filtered with filter paper Munktell grade 3 hw with a pore diameter of 110 mm (AHLSTROM, USA) to remove the excess of suspended solid residues. RW was then measured and classified as untreated-RW (U-RW). In contrast, treated-RW (T-RW) corresponded to the U-RW samples exposed to the Lac-Magnetite inside the Two-vertical-loop microreactor. 5 mg of Lac-Magnetite was introduced to the microreactor, as explained previously. 1 L of U-RW was pumped into the microreactor at a constant rate of 12 mL/h. The T-RW was then collected at the outlet of the microreactor.

Both samples, U-RW and T-RW, were analyzed to determine the Phenol content, Biochemical oxygen demand (BOD), Ammoniacal nitrogen, and Kjeldahl total nitrogen. The content of phenols was selected as they are susceptible to degradation by the immobilized Laccase molecules. Nitrogen was analyzed due the presence of amine groups in the immobilization linkers. Finally, the biochemical oxygen demand was estimated to indirectly examine the presence of living organisms.

The Removal Ratio for each of the parameters above was calculated according to Eq. (10), where the is the value obtained for each parameter in the untreated sample (U-RW), and is the value of the same parameter after treatment (T-RW).

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| --- | --- |
|  | Eq. (10) |

# Results and Discussion

## Impact of pH changes on the enzymatic activity

The impact of pH on the enzymatic activity was evaluated as shown in Figure 2. The optimal activity for the Free Laccase was evidenced under acidic conditions (Max. peak at pH 2). Similar optimal values between pH 2 - 4 were reported by other researchers [40][41]. In contrast, for pH values of 6 and above, the activity decreases to almost 0%. In the case of the bionanocomposites, the activity reached a peak at pH 4, followed by a considerable decrease for pH values of 6 and above. The change in the optimal pH in the immobilized enzyme to higher values is likely caused by an uneven concentration of the H+ and OH− ions between the support matrix and the bulk solution, leading to the observed shift in pH [33][34].

C:\Users\MULTINSA\Desktop\Imagenes paper 1\F2 pH dependency on the Enzymatic activity.tif

**Figure 2.** Impact of pH changes on the Enzymatic activity for: (●) Free-Laccase and (♦) Lac-Magnetite. Maximum and minimum errors are indicated with the error bars.

## Microreactor geometry design and simulation

Figure 3 shows the convergence plot for a one-loop microreactor. All the simulations were carried out with a minimum of with 60,000 mesh elements for the fluid domain to assure the system convergence.

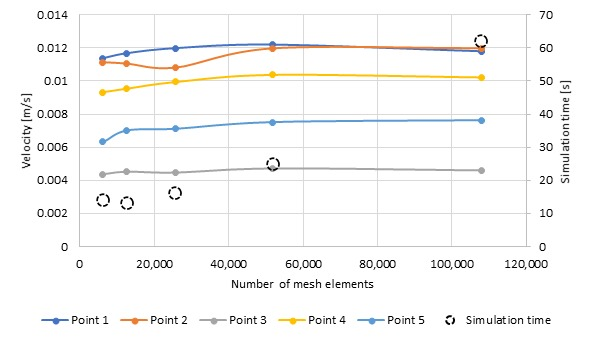


Figure 3. Mesh convergence analysis for the one-loop torus microreactor.

The velocity profiles and magnetic field fluxes for the 3 proposed devices are shown in Figure 4. Dead zones of low velocities (blue color) are observable in the 3 configurations, however, the two-vertical-loop microreactor shows a larger dead zone compared with the other 2 devices. This can be explained by the relatively important changes in height as the fluid passes through the loops. In addition, the fluid decelerates because of changes in the cross-sectional area of the microchannels. This velocity reduction is beneficial for biodegradation purposes as the interaction time of the bionanocomposites with the fluid is significantly exacerbated.

C:\Users\MULTINSA\Desktop\Imagenes paper juli cambiadas\F3 Velocity Profile completa.tif

**Figure 4.** Velocity profile for the three different configurations under study. A) One-loop, B) Two-horizontal-loop and C) Two-vertical-loop. Magnetic field flux for the three different configurations under study. D) One-loop, E) Two-horizontal-loop and F) Two-vertical-loop.

The magnetic field flux results show a uniform distribution around the magnet for the case of the single loop (**Figure 4**.A). For the two magnets arranged in a horizontal configuration (**Figure 4**.B), there is no interaction between the field lines of the magnets. Finally, the configuration of two-vertical-loop (**Figure 4** .C) showed the greatest field interaction, and consequently superior chances to retain the bionanocomposites.

The time evolution of the particle's transport within the one-loop microreactor was studied under varying intensities of the applied magnetic field (i.e., 0, 50, 100, 200, 300, 350, 500, and 1000 mT). The simulations were ran for a total time of 30 seconds with data collection at 1, 5, 15 and 25 s (See Figure 5). For these simulations, each particle trajectory is random and driven by the exerted drag and magnetophoretic forces. As the particles are attracted to the magnet and due to the interplay of involved forces in the loop, they follow a parabolic trajectory until finally sticking to the wall of the microreactor channel. For some particles, the magnetic field will not be enough to retain them within the loop. This unique trajectories have been exploited by others for the separation and manipulation of magnetic particles within microchannels [42][43]. In our case, the movement is enough to perturb the laminar flow, thereby generating mixing patterns that are useful to promote the intimate interaction of the nanoparticles with other components present in the solution. This was also evidenced by a better suspension of the nanoparticles as the magnetic field was increased.

*C:\Users\MULTINSA\Desktop\Imagenes paper juli cambiadas\F4 Particle distribution.tif*

Figure 5**.** Particle distribution in the One-Loop device at times: A) 1 s, B) 5 s, C) 15 s and D) 25 s.

Total retention of the particles was achieved for the fields of 500 and 1000 mT, as the particles remain statically attached to the walls of the microreactor. To support this result, the particle's loss and retention ratio were analyzed by counting the particles leaving the system through the microreactor's outlet during the 30 seconds of simulation (See Table 1). The experimentally applied magnetic field by the magnet was of 349.23 mT, which is close to the one simulated at 350 mT. In this case, the particles retention approached 96.83% while the loss ratio was 3.17%. The actual particles loss ratio obtained experimentally was between 13 - 20%, which was closer to the results obtained *in silico* at 300 mT where the retention ratio was 82.67%. In this case, the loss ratio approached 17.33%. The reduction in the actual strength of the magnetic field can be explained by the marked difference in the medium surrounding the fluid computationally (i.e., air) and the actual medium (i.e., PMMA walls), which attenuates the applied magnetic field.

Table 1.Retention ratio analysis at the microreactor's outlet after 30 seconds of simulation

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| --- | --- | --- |
| **Remanent flux density [mT]** | **Particle's loss ratio** | **Particle's retention ratio** |
| 0,00 | 93,67% | 6,33% |
| 50,00 | 91,17% | 8,83% |
| 100,00 | 85,00% | 15,00% |
| 200,00 | 56,00% | 44,00% |
| 300,00 | 17,33% | 82,67% |
| 350,00 | 3,17% | 96,83% |
| 500,00 | 0,00% | 100,00% |
| 1000,00 | 0,00% | 100,00% |

## Biodegradation studies

### Experimental tests for biodegradation of dyes

The possible dye retention on the surface of the microreactor´s walls was estimated by pumping the dye solution into the system in the absence of magnetite. The average retention obtained was 24.3%, 52.1%, and 26.2% for the three dye concentrations evaluated (i.e., 5, 10 and 20 mg/L). Figure 16 shows the average biodegradation of EBt as removal percentages of the dye for each device, treatment, and concentrations evaluated. Data are presented for the two types of analysis implemented in this work i.e., absorbance peak and area under the absorbance spectrum. For almost all the treatments, the maximum removal was achieved with the Lac-Magnetite except for one-loop at 10 mg/L and two-horizontal loop at 20 mg/L, where bare magnetite nanoparticles were more effective. We attributed this superior performance to the adsorption of the dye molecules on the surface of the nanoparticles. The maximum removal of dye was 98.74% for the two-vertical-loop microreactor. The average removal for Lac-Magnetite based on absorbance (545 nm) (Figure 6.A) was 91.08%, 74.38% and 96.21% for the one-loop, two-horizontal-loop, and two-vertical-loop microreactors, respectively. The dye removal for the same treatment but based on the area under the absorbance spectrum (Figure 6.B) approached 85.62%, 62.44%, and 93.56% for the three microreactors. Taken together, these results strongly suggest that the microreactor with the best performance in terms of the dye removal was the two-vertical-loop one. Also, in the case of the Lac-Magnetite treatment, the overall (absorbance peak and area under the absorbance spectrum) removal efficiencies for this microreactor approached 98.05%, 93.87%, and 92.74% for the low, medium, and high concentrations of dye evaluated.

C:\Users\MULTINSA\AppData\Local\Microsoft\Windows\INetCache\Content.Word\F5 Removal Percentage.tif

**Figure 6.** Removal Percentage of Eriochrome Black T Dye for the three studied microreactor configurations at low: 5 mg/L, medium: 10 mg/L and high: 20 mg/L concentrations of dye. A) as estimated by absorbance peak. B) as estimated by absorbance area. Maximum and minimum errors are indicated with the error bars. Percentage of oxidized Phenol in Artificial Wastewater for the two-vertical-loop microreactor at, medium and high concentrations of phenol. C) as estimated by absorbance peak. D) as estimated by absorbance area. Maximum and minimum errors are indicated with the error bars.

### Experimental tests for biodegradation of phenol in artificial wastewater

Due to the performance of the two-vertical-loop microreactor, we conducted biodegradation tests for the biodegradation of the phenol present in the artificial wastewater. During enzymatic catalysis, the laccase molecules oxidize phenol molecules to form free radicals, which is followed by their decarboxylation and demethylation[25][44].

**Figure 6** shows the average percentage of oxidized phenol for both types of analysis (i.e., absorbance peak and area under the absorbance spectrum) and for each treatment and concentration evaluated. The negative percentages observed in the absence of the bionanocomposites can be attributed to physical adsorption on either the walls of the microreactor or on the nanoparticles. Importantly, during experimentation we evidenced a change in the color of the samples (from transparent to yellow) after the treatment, most likely caused by interactions with the manufacture material or the used adhesive. In all cases, the Lac-Magnetite treatment showed positive percentages of oxidized phenol, which confirmed the oxidation carried out by the immobilized Laccase molecules. The maximum oxidation, 79.89%, was obtained for the Lac-Magnetite treatment at the lowest dye concentration.

### Treatment with a large volume of real wastewater

A large volume real wastewater sample was treated in the two-vertical-loop microreactor in the presence of Lac-Magnetite. Table 2 shows the collected environmental parameters for the wastewater samples before (U-RW) and after (T-RW) treatment. The removal ratio for phenol approached 17.86%, achieving a final concentration of 16.10 mg/L, which is close but still greater than the maximum permissible level for drinking water in the U.S. i.e., of 11 mg/L [45] as imposed by the United States Environmental Protection Agency (EPA) regulations. However, this reduction is promising and encouraged us to continue exploring further improvements to the microreactors in future contributions. Moreover, no other microreactor has been identified in the literature capable of continuous operation for phenol remediation employing large volumes of real wastewater that also offers the possibility for in-line and real-time monitoring. In this regard, recent reports indicate that most successful phenol remediation strategies of real wastewater rely on batch biodegradation processes [46]. For instance, Garg et al. reported the use of low-purity peroxidases extracts for the removal of phenol from textile industry wastewater in a batch process with an efficiency of 94.95 ± 0.82%. Additionally, the same process led to 91.49 ± 1.54% removal efficiency in leather industry wastewater [46]. Hayati et al. reported a removal ratio of 85.9% (TOC) and 92.1% (COD), with GZnTi nanocomposites [47].

As there are amine groups present in the molecules used as linkers to complete the immobilization process, the nitrogen content becomes a parameter of interest. According to the results of two different techniques, i.e., Ammoniacal nitrogen and Kjeldahl total nitrogen, the Nitrogen removal ratio was 16.67% and 20.16%, respectively. This indicates that the functionalization of the bionanocomposites was successful. Additionally, as the emission limit value (ELV) for nitrogen in wastewater by the regulation presented by the United States Environmental Protection Agency (EPA), is 10 mg/L and 15 mg/L [48], for Ammoniacal nitrogen and Kjeldahl total nitrogen respectively; the treatment is likely helping in the nitrogen removal (Final concentrations of 0.50 and 9.90 mg/L) as well. In the case of the biochemical oxygen demand (BOD), the removal ratio was 10.39%, which allowed us to conclude that the treatment might be useful to prevent the growth of microorganisms during treatment of wastewaters.

Table 2.Characterization of the large volume samples of real wastewater treated with the developed bionanocomposites

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Parameter** | **Sample** | | | **Units** |
| **U-RW** | **T-RW** | **Removal Ratio** |
| Phenols | 19.60 | 16.10 | 17.86% | mg Phenol /L |
| Biochemical oxygen demand (BOD) | 385.00 | 345.00 | 10.39% | mg O2/L |
| Ammoniacal nitrogen | 0.60 | 0.50 | 16.67% | mg N /L |
| Kjeldahl total nitrogen | 12.40 | 9.90 | 20.16% | mg N /L |

The initial phenol concentration of the real wastewater sample was 19.60 mg/L, which is close to the highest concentration evaluated in the case of biodegradation of dyes with model wastewaters. Also, the experiments with real wastewaters were conducted in the presence of 5 mg of Lac-Magnetite. Consequently, an equivalent number of cycles to treat the total volume of sample in each case can be estimated to be roughly 200. To our knowledge, there are no reports of microfluidic systems tested at such high number of cycles. Nonetheless, Hayati et al. showed that after 6 cycles of treatment, phenol degradation efficiency remained in the range of 90–100% [47], and Abdollahi et al. reached about 52% phenol reduction efficiency after 6 cycles of use [49].

# Conclusions

Some of the main phenol bioremediation issues of the wastewaters in advanced bioreaction systems include the difficulty to maximize the efficiency of the biodegradation processes, the presence of dead volumes, and the limited mixing of reagents. To overcome these issues, three different toroidal microreactors equipped with a permanent magnet, were designed, and manufactured at low cost to perform the phenol bioremediation with laccase-based bionanocomposites that respond to magnetic flieds. The synthesized bionanocompound exhibited better performance under slight acid conditions, i.e., pH 4 to 4.5.

On the other hand, the torus microreactor configuration equipped with a magnetic field source prolongs the residence time of the nanoparticles within the system without inducing detrimental perturbations in the mixing patterns. This leads to a longer contact time between the nanoparticles and other substances present in the medium. Furthermore by the time evolution of the particle's transport analysis it was proved that the presence of the magnetic field generates a parabolic movement on the particles, breaking the stability of the laminar flow creating a mixing phenomenon that promote the interaction of the solution to be treated with the bionanocompound. Other torus reactors use mechanical propellers to improve mixing (ref); however, the proposed microreactors implemented fixed magnetic components to achieve this item, reducing energy consumption in the long term.

Similarly, by the multiphysics simulations was determined that the two-vertical-loop microreactor is having a greater area of interaction, dead zone area, with the magnetic field, allowing a longer residence time of the particles treating the solution and therefore a better degradation of the phenol molecules. This was ascertained by the results found in the experimental test for biodegradation of dyes, where the overall average removals for this microreactor with a Lac-Magnetite treatment were 98.05%, 93.87%, and 92.74% for the 3 dye concentrations evaluated. Similarly, the biodegradation test for the phenol present in the artificial wastewater led to a maximum oxidation level of 79.89% with the Lac-Magnetite treatment at the lowest dye concentration evaluated. Finally, the treatment of a large volume of real wastewater led to a 17.86% phenol removal ratio, which is equivalent to performing 200 work cycles of biodegradation. This experiment represents a first step towards defining the criteria for a large-scale bioremediation of wastewater enabled by advanced microfluidic devices.

# Discussion

The results of the present research work pave the way for the scaling-up of the bioremediation processes by either enabling recirculation strategies or by parallelizing multiple devices in a continuous operation. As the permanent magnet is retaining the bionanocomposites it can be removed for cleaning purposes and subsequently reinstalled. In this type of operation scheme. It is of paramount importance to determine lifetimes for the bionanocompound, the device, the pumping system, and the magnet. This is critical to assure a stable operation, reusability and to define the maintenance cycles. An additional important unanswered question is whether the bionanocomposites can improve their removal efficiencies by operating at temperatures above conventional mesophilic conditions. This will require further adjustment and testing to assure the devices’ absence of leakages and material stability in the long run. Finally, we believe that it is worth to explore other microreactor geometries where a dynamic array of magnets can be adjusted to maximize the magnetic gradient and other geometry mixing approaches.

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