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Improving the effectiveness of safe cyanotoxin removal from drinking water using magnetic iron oxide nanoparticle-loaded carbon composites

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ABSTRACT

Climate change and eutrophication have increased cyanobacterial blooms, raising exposure to cyanotoxins in drinking water. This study investigates efficacy improvement of two magnetic nanostructured particles with different adsorbent materials, mesoporous carbon (P-Mes) and Merck activated carbon (P-MAC), for cyanotoxin removal. Safety of these materials is also investigated in extreme conditions of physical deterioration. Their efficacy was tested using pre-cleaning with organic solvents, different toxin concentrations, particle/volume ratios and toxin mixtures. Acetonitrile and ethanol pre-cleaning improved toxin adsorption in both particles. P-Mes particles demonstrated high adsorption for high-molecular-weight toxins, achieving 97 % removal of microcystin-LR at a particle-to-water ratio of 1:750 μL, and were also effective removing seven other microcystins and saxitoxin. P-MAC particles exhibited superior performance for low-molecular-weight toxins, achieving removal rates of 98 %, 91 % and 83 % for anatoxin-a, saxitoxin, and cylindrospermopsin respectively, at a particle-to-water ratio of 1:1.5 mL. Additionally, combination of particles or toxin mixtures did not inhibit their effectiveness. In vitro toxicity assessments using pulverized particles to increase possible release of toxic components indicated no cytotoxicity in SH-SY5Y, CACO-2, HepG2, and CAKI-1 lines, and no alteration of CACO-2 monolayers electrical resistance. Overall, materials preconditioning, particle-to-volume ratio adjustment, and selection of appropriate composite combination enhances removal of nine toxins.

1. Introduction

Cyanotoxins are natural compounds produced by cyanobacteria, which have emerged as significant environmental pollutants with profound ecological and human health implications [1]. The proliferation of cyanobacterial blooms, fuelled by nutrient enrichment and climatic factors, has intensified concerns regarding the prevalence and persistence of cyanotoxins in aquatic ecosystems worldwide [2].

Many cyanotoxins have been described with different origins and chemical structures [3]. Currently, the more common groups of cyanotoxins are microcystins (MCs), which are hepatotoxins; cylindrospermopsin (CYN), currently considered a cytotoxin; and anatoxin-A (ATX-A) and saxitoxins (STXs), among the neurotoxins [4]. Remarkably, although the four groups have several analogues, in fresh water over 300 different MCs have been identified due to variable combinations of amino acids in their heptapeptide structure, even though the great

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majority of these molecules are rarely encountered [5]. MCs are globally distributed and the abundance of their congeners depends on the sampling area, with the analogues MC-LR, desmethyl-MC-LR (des-MC-LR), MC-RR, MC-LA, MC-LF, MC-LY and MC-YR being among the most abundant [6,7].

Over the years, there has been increasing evidence of the toxic effects of these substances on humans and animals [8,9]. Therefore, research on the identification and removal of cyanotoxins from water for the improvement of public health has been intensified in the last decades [10–12]. Current water treatment techniques focus primarily on the removal of cyanobacterial biomass, which removes intracellular toxins along with bacteria; however, elimination of free cyanotoxins, which can also be present in water due to bacterial lysis, is a complex challenge [13]. Some studies support the efficacy of oxidizing chemicals, which are already being used in conventional purification stations, for removing free cyanotoxins from water [14,15]. Nevertheless, the generation of other harmful compounds such as trihalomethanes [16] or the need for additional techniques like UV radiation [17] contribute to the high costs and safety concerns of these treatments. Another method described in the literature involves the use of bacteria capable of degrading cyanotoxins in water [18,19]. This approach, nevertheless, requires extended periods and may disrupt the ecosystem if these bacteria are released to the surrounding environment. These concerns make these methods unsuitable for the safe and practical removal of cyanotoxins from water in treatment stations.

In recent years, a number of different materials have been tested for the adsorption of cyanotoxins, as graphene oxide [20], organic polymers [21], waste biomass [22], protonated mesoporous graphitic carbon nitride [23], biochars [24] or carbon [25]. However, the large and complex nature of cyanotoxins makes it challenging to find a suitable adsorbent for all toxins. Moreover, adsorbents can be used in different presentations, from nanoparticles, nanotubes, fibers or beads to magnetic nanostructured composites, with different characteristics or advantages/disadvantages for practical use in treatment plants. Additionally, most studies lack data on potential residues that could be released to the water and be harmful to people. In a previous publication [26], cyanotoxin removal with adsorbent materials in a presentation of superparamagnetic particles composed of a porous biopolymeric matrix containing magnetic nanoparticles was explored. Preparation of magnetite nanoparticles by co-precipitation provides cost-effective composites due to the simplicity, scalability, and low material and energy requirements needed for this magnetic nanoparticle fabrication method [27]. Furthermore, superparamagnetic properties ensure dispersion of magnetic particles in a liquid environment for adequate contact with the contaminant in solution, and posterior efficient removal with a magnet. In this study, the best adsorption materials were mesoporous carbon (P-Mes) and Merck activated carbon (P-MAC) from a selection of ten adsorbents. For practical use of adsorbent methods, not only efficacy of toxin removal is relevant, but also performance must be maintained in the presence of several toxins simultaneously.

In this study, the conditions for improving the adsorption efficiency of activated carbon and mesoporous carbon composites loaded with magnetic iron oxide nanoparticles were explored, as well as removal performance for ten different cyanotoxins, CYN, ATX-A, saxitoxin and seven microcystins, and for toxin combinations. In addition, in vitro toxicity in conditions of extreme physical damage of the nanostructured composites was evaluated to extend toxic characterization of these materials.

2. Material and methods

2.1. Chemicals and reagents

Acetonitrile (AcN), acetic acid and methanol (MeOH), UHPLC quality, were supplied by Panreac Quimica S.A. (Barcelona, Spain). Hydrogen peroxide (H_2O_2) 33 % was from Scharlab (Barcelona, Spain) and ammonium formate and formic acid were from Merck (Madrid, Spain). Water was purified using a Millipore Milli-Q Plus system (Millipore, Bedford, MA). Durapore centrifugal filters ultrafree-MC (0.22 μm pore size) were from Millipore and nylon Spin-X® centrifuge tube filters (0.22 μm pore size) were from Corning (New York, USA). Certified reference materials of MC-LR, MC-RR, MC-LY, MC-LF, MC-YR, CYN and ATX-A were provided by Cifga S.A. (Lugo, Spain), MC-LA from Eurofins Abraxis (USA) and STX and des-MC-LR from National Research Council of Canada. Analytical grade MC-LR, MC-RR, MC-LA, MC-YR, MC-LY and MC-LF were provided by Enzo Biochem (USA) and analytical grade CYN was from Santa Cruz Biotecnology (Germany). MC-LR and CYN were dissolved in water, MC-RR in methanol 80 %, MC-YR, MC-LY and MC-LA in ethanol and MC-LF in methanol, as indicated by the manufacturer, stock solutions of all toxins were prepared at a concentration of 100 μg/mL.

All chemicals used for the nanostructured particles preparation were of analytical grade. Powder extra pure activated carbon was obtained from Merck (Darmstadt, Germany), ferric chloride ($FeCl_3{\cdot}6H_2O$) from Alfa Aesar (Madrid, Spain), and surfactant Tween 20 from Fluka (Steinheim, Germany), calcium chloride ($CaCl_2$), ammonium hydroxide (NH_4OH, 28 %), triblock copolymer Pluronic P123 (PEO20-PPO70-PEO20), tetraethyl orthosilicate (TEOS, 98 %), sucrose (99 %), sulphuric acid (H_2SO_4, 95–97 %), hydrofluoric acid (HF, 48 %), hydrochloric acid (HCl, 37 %), and sodium alginate were purchased from Sigma Aldrich (Saint Louis, MO, USA).

2.2. Adsorbent magnetic nanostructured particles

Magnetic nanostructured particles were prepared by extruding a mixture of sodium alginate, adsorptive components and magnetite (Fe_3O_4) nanoparticles into a calcium chloride coagulation bath. Magnetite nanoparticles were synthesized by the reverse coprecipitation method and characterized as described in [27]. The adsorbent blend was stirred at room temperature for 4 h, then delivered dropwise at 1.5 mL/min using a New Era NE-300 syringe pump into a bath containing 0.13 M $CaCl_2$ and 450 μL Tween 20 under magnetic stirring at 500 rpm. Magnetic nanostructured particles were formed immediately upon entry and were left to harden for 20 min. Finally, the particles were rinsed with distilled water and stored at 4 °C in Milli-Q water.

P-Mes particles were composed by Fe_3O_4 (17.9 %), sodium alginate (51.3 %) and CMK-3 mesoporous carbon (30.8 %) and their dry average weight and diameter were 6.1 mg and 2 mm respectively. CMK-3 was prepared as in [28]. On the other hand, P-MAC particles were formed by Fe_3O_4 (7.92 %), Merck activated carbon (55.2 %) and sodium alginate (36.8 %) and have a dry average weight and diameter of 6.0 mg and 3 mm respectively.

2.3. Adsorption experiments

Adsorption effectiveness was assessed after incubation of 4.5 mL of cyanotoxin solution in milli-Q water (pH 5.7) with 3 nanostructured particles (average of 4 mg of adsorbent composites/mL) for 2 h under constant shaking at an average temperature of 21 °C, in glass tubes for all toxins except STX. For STX the experiment was performed in the same way but using polystyrene centrifuge tubes. The initial toxin concentration was set at 60 μg/L for MC-LR, CYN, and STX, and at 10 μg/L for ATX-A, except in experiments where different toxin concentrations were evaluated. In those experiments, concentrations of 180, 150, 120, 90, 60, 30, and 10 μg/L were used for MC-LR; 360, 300, 240, 180, 120, 60, and 20 μg/L for CYN; and 40, 33.3, 26.6, 20, 13.3, 6.6, and 2.2 μg/L for ATX-A. Experiments directed to test differences among particle batches were performed following this protocol, with only one toxin concentration, shortly after particle fabrication. All cyanotoxins were tested separately unless otherwise specified. The particle/volume of toxin solution ratio indicated above was modified in a set of experiments from 1:1.5 mL (average of 4 mg of adsorbent composites/mL) to values of

1:0.75 mL (8 mg of adsorbent composites/mL), 1:0.5 mL (12 mg of adsorbent composites/mL), 1:0.375 mL (16 mg of adsorbent composites/mL) and 1:0.3 mL (20 mg of adsorbent composites/mL), corresponding to initial amounts of toxin in solution/particle ratios of 90 ng MC-LR or CYN/particle or 15 ng ATX-A /particle, 45 ng MC-LR or CYN/particle or 7.5 ng ATX-A/particle, 30 ng MC-LR or CYN/particle or 5 ng ATX-A/particle, 22.5 ng MC-LR or CYN/particle or 3.75 ng ATX-A/particle and 18 ng MC-LR or CYN/particle or 3 ng ATX-A/particle respectively. In any case, samples of 120 µL were collected at the indicated times and quantified using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) for all cyanotoxins, except for STX, which was quantified using high-performance liquid chromatography coupled to fluorometric detection (HPLC-FLD). For each condition at least 3 independent experiments were performed. No loss of particle integrity was observed during toxin adsorption experiments. Changes of these standard experimental conditions that are required for the purpose of the experiment are reported in the Results section.

2.4. MCs, CYN and ATX-A quantification by liquid chromatography coupled to mass spectrometry

MCs, CYN and ATX-A present in water samples were quantified using UHPLC coupled to a mass spectrometer as in [26]. Briefly, after filtration, samples were held at 4 °C and 5 µL were injected onto an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm) at 35 °C. Separation was performed using water (A) and acetonitrile (B) mobile phases, each with 0.05 % formic acid, at 0.4 mL/min with a chromatography program starting from 2 % B (4 min), a gradient from 2 % B to 85 % B (4.5 min), holding at 85 % B (4 min), then returned to 2 % B for 1 min and re-equilibration for 4 min. Samples were analysed immediately or stored at −20 °C.

Regarding mass spectrometry, the optimized mass transitions and electrospray ionization (ESI) source parameters for each toxin are shown in Table 1. When the experiment includes samples with a combination of toxins, the ESI conditions for the compound with lower sensitivity were applied for all toxins. Toxin quantification was performed as in [26]. Additional calibration points were introduced when necessary.

2.5. Liquid chromatography coupled to fluorometric detection

Water samples containing STX were quantified by a modified Lawrence method [29] using a HPLC system coupled to a fluorescence detector. The chromatographic setup was from Shimadzu (Izasa, Barcelona, Spain) and included a binary LC-10ADVP pump system, autoinjector SIL-20 AC, column oven CTO-20 AC, a fluorescence detector RF-10AXL and the system controller CBM-20 A.

Before conducting the analysis, oxidation of samples and calibration solutions was performed by adding 100 µL of sample/calibration solution to a mixture of 250 µL of 1 N NaOH and 25 µL of 3.3 % H_2O_2. After 2 min, 20 µL of acetic acid were added. Then, this solution was filtered using nylon centrifuge filters and the samples were placed in the HPLC autosampler at a temperature of 4 °C. Sample injection volume was 25 µL. Liquid chromatography separation was performed using a HPLC SUPELCOSIL LC-18 column (15 cm × 4.6 mm, 5 µm, Merck) at 35 °C. The mobile phases A and B were 0.1 M ammonium formate in water and 0.1 M ammonium formate in 5 % AcN respectively, both adjusted to pH 6 with 0.1 M acetic acid. Chromatography was performed at a flow rate of 1 mL/min with a gradient of mobile phase B as follows: lineal increase from 0 % to 5 % for 5 min, then to 70 % over 4 min, linear decrease to 0 % in 2 min and final re-equilibration at 0 % for 9 min. A fluorometric detector was used to detect oxidized STX with excitation and emission wavelengths at 340 nm and 395 nm respectively.

Table 1
UHPLC-MS/MS optimized parameters for quantifying MCs, CYN and ATX-A samples.

Mass transitions and MS/MS conditions

Compound	Precursor	Product ion	CE	Fragmentor	CAV	Polarity
MC-LR	995.56	135	80	220	1	Positive
	498.28	135	8	105		
		861.4	8			
MC-RR	519.79	103	84	155	2	Positive
		155	28			
MC-LF	986.53	135	60	220	1	Positive
		478.2	20			
MC-LY	1002.52	135.1	60	215	4	Positive
		375.1	28		3	
MC-LA	910.49	135	68	195	1	Positive
		107.1	92		2	
MC-YR	523.27	135.1	8	85	2	Positive
		911.4	8		1	
dm-MC-LR	491.27	135	8	85	1	Positive
		847.4	8			
CYN	416.13	194.2	36	160	1	Positive
		336.1	20			
ATX-A	166.13	43.2	28	90	1	Positive
		91.1	28			

ESI parameters

Compound	SGT (°C)	SGF (L/min)	GT (°C)	GF (L/min)	Neb. (psi)	Cap (V)	NV (V)
MC-LR	250	12	280	8	30	3500	1500
MC-RR	400	11	250	8	30	3000	1000
MC-LF	400	11	250	8	30	4500	0
MC-LY	250	12	320	11	35	3500	1500
MC-LA	250	11	230	9	30	3500	1500
MC-YR	300	12	230	5	25	3500	1500
dm-MC-LR	400	11	260	6	35	5000	1500
CYN	250	12	260	12	35	5000	1500
ATX-A	400	9	260	6	25	4500	1500

CE: collision energy; CAV: cell accelerator voltage; SGT: sheath gas temperature; SGF: sheath gas flow; GT: gas temperature; GF: gas flow; Neb.: nebulizer; Cap: capillary; NV: nozzle voltage.

2.6. Cytotoxicity detection in cell cultures

CAKI-1, SH-SY5Y, HepG2 and CACO-2 cell lines maintenance and cytotoxicity assays were performed as in [26]. Briefly, cytotoxicity was assessed in 96-well clear-bottom white plates (Corning, USA) using the AlamarBlue assay (Thermofisher, USA). Cells were seeded and allowed to adhere for 24 h. Concentrations ranging from 4 mg/mL to 30 µg/mL of pulverized P-Mes and P-MAC were kept in agitation for 24 h in medium or water. Then, samples were centrifugated at 3000 rpm for 5 min and the cells were treated with the supernatant. Test wells received 100 µL of particle-exposed water (final concentration 50 %) or the culture medium was replaced with 180 µL particle-exposed medium, and finally 10 % AlamarBlue was added. Milli-Q water and untreated medium controls, in addition to a 1 mg/mL saponin death control, were performed simultaneously. Fluorescence (excitation of 530 nm and emission of 590 nm) was recorded at 4, 8, 12, 24 and 48 h using a plate reader (Synergy, Biotek).

2.7. Trans-epithelial electric resistance measurement assays

CACO-2 cells were seeded in a Milli-cell hanging culture insert with a 0.4 µm pore size polyethylene terephthalate membrane bottom (Millipore Corporation, Bedford, MA, USA), placed in a well of a 12-well plate, at a density of 60,000 cells, and cultured until differentiation over 21 days at 37 °C [30]. The tested samples were water exposed to particles or medium in contact with pulverized particles. The samples with pulverized particles were centrifuged, and the cells were treated with the

supernatant. Water samples were added to the apical surface at a final concentration of 50 % and medium samples were added to inserts after removal of cell culture medium, both at a final volume of 500 μL. Controls of medium or Milli-Q water without particles were also conducted. Transepithelial electrical resistance (TEER) was measured in non-supplemented MEM medium using a Millicell-ERS Multimeter from Millipore Corp. (Bedford, MA, USA) before and after treatment. Resistivity was calculated according to the following formula: Resistivity (Ω/cm^2) = TEER (Ω)/Area (cm^2). Only inserts with cultures that displayed TEER higher than 300 Ω before the addition of the sample were used.

2.8. Statistical analysis

Statistical analyses were performed as described in [26]. Briefly, data were obtained from 3 independent experiments (performed in duplicates for adsorption experiments and in triplicates for cell-viability assays). Statistical significance was evaluated using one-way ANOVA ($p < 0.05$).

3. Results

3.1. Effect of particle production batch and preconditioning on adsorption experiments

Two production batches of P-Mes and P-MAC nanostructured particles were supplied. To characterize the homogeneity of the nanostructured particles production, a comparison of the adsorption characteristics of both batches was performed. Three nanostructured particles of each batch were kept in contact with 4.5 mL of a solution of 60 μg/L MC-LR and CYN and 10 μg/L ATX-A (corresponding to an initial amount of toxin in solution/particle of 90 ng MC-LR and CYN and 15 ng ATX-A/particle). Statistical differences were observed in the adsorption efficiency of MC-LR to P-Mes particles between batches, with a 22.7 % decrease in adsorption in the second batch vs the first one (Table 2). Similarly, ATX-A adsorption was also reduced in the second P-Mes batch (Table 2). On the contrary, the second batch of P-MAC particles demonstrated a significant increase in MC-LR adsorption, although the adsorption percentage remained lower than that of P-Mes particles. In contrast, the adsorption of CYN and ATX-A to P-MAC particles did not show statistical differences between batches.

To evaluate particle pretreatment as a possible alternative to improve adsorption performance, particle cleaning using different solvents was conducted prior to exposing the materials to a cyanotoxin solution. For this purpose, water, ethanol, and 75 % acetonitrile were used as solvents, each applied at a ratio of 1 mL per particle for 60 min, followed by three washes with the same volume of Milli-Q water. If any improvement was observed after the first wash cycle, the cleaning procedure was repeated. Water cleaning did not significantly improve the adsorption of any particles. However, washing P-Mes particles twice with ethanol significantly increased MC-LR adsorption efficiency from 43.87 ± 4.5 % to 60.47 ± 3.7 %, while 75 % acetonitrile did not produce a significant improvement. On the other hand, a treatment involving four wash cycles with 75 % aqueous acetonitrile increased P-MAC adsorption of CYN from 41.95 ± 3.1 % to 85.15 ± 2.63 %. The 4-cycle 75 % acetonitrile pretreatment showed a slightly higher improvement of CYN removal than 4-cycle ethanol pretreatment, although they were not statistically different. Statistical power should be increased to finally determine if any of these two treatments provides better efficiency of toxin removal. Preliminary experiments have been conducted demonstrating that the particle-to-solvent ratio could be reduced, though further optimization could be required for final application. In order to work in the best conditions possible, the next experiments with P-Mes and P-MAC were performed with 2-cycle ethanol and a 4-cycle 75 % acetonitrile pre-conditioning respectively.

Table 2
Adsorption of MC-LR, CYN and ATX-A toxins to the first and second batches of P-Mes and P-MAC. Toxin adsorption was tested using solutions of 60 μg/L MC-LR and CYN and 10 μg/L ATX-A separately, with a particle-to-water volume ratio of 1:1.5 mL. Toxin solutions were in contact with the particles for 2 h. Results are expressed as percentage of removal considering initial and final concentration (mean ± SD, $n = 3$). *: Statistically significant versus first batch.

	P-Mes		P-MAC	
	First batch	Second batch	First batch	Second batch
MC-LR	56.76 ± 3.82 %	43.87 ± 4.51 % *	1.78 ± 3.11 %	16.71 ± 2.70 % *
CYN	21.50 ± 1.31 %	19.05 ± 3.60 %	43.00 ± 2.74 %	41.95 ± 3.13 %
ATX-A	42.78 ± 2.76 %	25.46 ± 2.97 % *	98.76 ± 0.18 %	98.68 ± 0.33 %

3.2. Toxin adsorption to P-Mes and P-MAC pre-conditioned particles

In order to evaluate more extensively the efficiency of P-Mes and P-MAC preconditioned particles, removal from water of 10 different cyanotoxin molecules was tested. Experiments were conducted adding prewashed P-Mes or P-MAC particles to different solutions of 60 μg/L of CYN, STX or seven MC analogues and 10 μg/L of ATX-A. The toxin remaining in solution after 2 h of exposure to the particles was quantified by UHPLC-MS/MS for all toxins, except for STX, that was quantified by HPLC-FLD. The results are expressed as percentage of toxin removed from solution considering final and initial concentrations and as weight of toxin removed per g of particle. A tube without particles was included in every experiment to account for toxin degradation or binding to container walls and no toxin losses in this control were detected.

Initially, adsorption of representative toxins of four cyanotoxin classes was tested. Regarding MC-LR, P-Mes particles achieved the highest adsorption removal 2 h after being added to the contaminated solution, surpassing the performance of P-MAC particles (Table 3). Conversely, P-MAC particles showed the best removal efficiency for CYN and ATX-A, which are compounds with a lower-molecular-weight than MCs, achieving >85 % removal for both toxins 2 h after their addition to the contaminated solution (Table 3). In contrast, P-Mes particles have not demonstrated good adsorption of these two toxins after the same time. Finally, STX achieved the second-highest adsorption rate after ATX-A, with a removal efficiency exceeding 90 % using P-MAC particles, although for this toxin no statistical differences were observed when compared to removal efficiency of P-Mes particles (Table 3).

Since nearly complete adsorption was achieved for STX in 120 min, an adsorption kinetics experiment was conducted to evaluate the possibility of reducing exposure time, similarly to what was found for ATX-A in previous studies [26]. In this experiment, samples were taken at 10, 30, 60, and 120 min after the addition of particles to the contaminated solution with 60 μg/L STX. The results showed a statistically significant increase in adsorption for both particle types at all time points,

Table 3
P-Mes and P-MAC adsorption of cyanotoxin representative toxins MC-LR, CYN, ATX-A and STX. Toxin adsorption was tested using solutions of 60 μg/L MC-LR, CYN and STX and 10 μg/L ATX-A separately with a particle-to-water volume ratio of 1:1.5 mL. Results are expressed as percentage of removal and as amount of toxin removed per gram of particles considering initial and final concentrations (mean ± SD, $n = 3$). *: Statistically significant versus P-Mes adsorption ($p < 0.05$).

Toxin	P-Mes		P-MAC	
	%	μg/g	%	μg/g
MC-LR	60.47 ± 3.70	6.75 ± 0.38	15.71 ± 3.50*	2.09 ± 0.38*
CYN	21.73 ± 3.48	3.86 ± 0.21	85.15 ± 2.63*	13.02 ± 1.08*
ATX-A	26.09 ± 4.16	1.32 ± 0.05	98.00 ± 0.34*	3.28 ± 0.60*
STX	81.68 ± 3.16	12.71 ± 0.53	90.73 ± 4.40	14.1 ± 0.61

indicating no particle saturation. Additionally, no statistically significant differences were observed between the two particle types at any time (Fig. 1).

Because P-Mes particles were better removing MC-LR, this material was also tested for the removal of other 6 microcystin analogues, MC-RR, MC-LF, MC-LY, MC-LA, Des-MC-LR and MC-YR, from water. The results indicate that MC-RR is removed most effectively, followed by MC-LR and MC-YR. Moderate adsorption was observed for MC-LY, MC-LF and des-MC-LR. Finally, adsorption of MC-LA was much lower (Table 4).

3.3. Effect of adsorption conditions on P-Mes and P-MAC efficiency

3.3.1. Combination of cyanotoxins

Because many species of cyanobacteria can produce more than one cyanotoxin, toxin adsorption of P-Mes and P-MAC in the presence of a combination of toxins has been evaluated. The effect of combining the representative compounds of three different cyanotoxin classes, MC-LR, CYN and ATX-A, on the adsorption of both particles was tested exposing 3 nanostructured particles for 2 h to 4.5 mL solutions with one of these toxins or combinations of two or three toxins. Initial concentrations were 60 μg/L for MC-LR and CYN and 10 μg/L for ATX-A. There were no statistical differences between the adsorption in solutions with toxins mixed in different combinations and the adsorption of the toxins alone in either of the two particles (Fig. 2).

In addition, to evaluate the efficiency of adsorption to the particles in the presence of several toxins of the same class, which have similar molecular structures, a 4.5 mL solution with seven microcystin analogues, in parallel to separate solutions of each toxin, were keep in contact with 3 P-Mes particles for 2 h. Toxin concentration was 60 μg/L for all MCs. P-Mes particles showed no statistical differences between removal percentages of MCs alone and in combination with other 6 MCs (Fig. 3).

3.3.2. Combination of nanostructured particles

The possibility of improving global toxin removal of the three toxins MC-LR, CYN and ATX-A, using both nanostructured particles simultaneously was evaluated by combining P-MAC and P-Mes particles in the same solution. First, a working solution of 60 μg/L MC-LR and CYN and 10 μg/L ATX-A was prepared, and then 4.5 mL was transferred into each of 3 glass tubes. Subsequently, 3 particles of P-Mes were added to one tube, 3 particles of P-MAC to another tube and a combination of 3 particles of P-Mes and 3 particles of P-MAC to the last tube. The adsorption was assessed by quantifying initial and final toxin concentrations in solution for each condition. The percentage of toxin removal was similar for CYN and ATX-A when P-MAC particles were used alone or in combination with P-Mes. Similarly, MC-LR removal was not statistically different in P-Mes particles vs the mixture of P-Mes and P-MAC materials (Fig. 4).

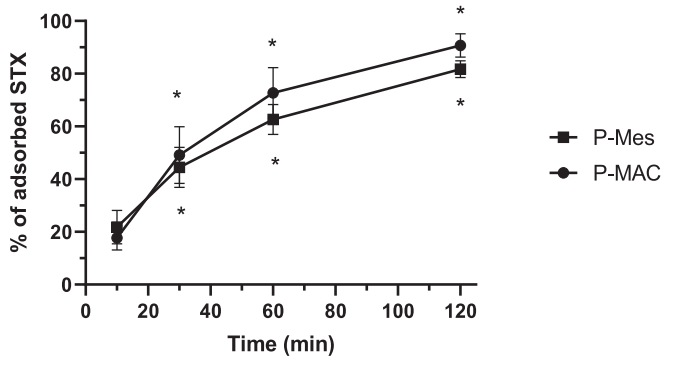

Fig. 1. Adsorption kinetics of STX by P-Mes and P-MAC nanostructured particles. Adsorption was evaluated using a solution of 60 μg/L STX in water exposed to P-Mes and P-Mac particles in a particle-to-water volume ratio of 1:1.5 mL. Samples were collected before and at 10, 30, 60 and 120 min after adding the particles (mean ± SD, n = 3). * Statistically significant vs previous time point ($p < 0.05$). No statistical differences between particles were found at any time.

Table 4
P-Mes adsorption of seven MC analogues. Experiments were conducted using solutions of 60 μg/L of each analogue separately with a particle-to-water volume ratio of 1:1.5 mL. Results are expressed as percentage of removal and as amount of toxin removed per gram of particles considering initial and final concentrations (mean ± SD, n = 3). Statistically different vs *all other MCs, ** all except LY, LF and des-LR, # all except LR or YR ($p < 0.05$).

MC analogue	P-Mes adsorption	
	%	μg/g
MC-LR	60.47 ± 3.7	6.75 ± 0.38
MC-RR	84.98 ± 1.97*	8.33 ± 0.19
MC-LF	42.18 ± 5.21**	4.44 ± 0.55
MC-LY	42.81 ± 1.86**	4.65 ± 0.20
MC-LA	12.93 ± 7.41*	1.60 ± 0.91
Des-MC-LR	41.62 ± 3.38	5.72 ± 0.41
MC-YR	58.74 ± 3.82#	5.12 ± 0.33

3.3.3. Particle/ volume of toxin solution ratio

The appropriate ratio of particles per volume of solution was investigated to facilitate future integration into a water treatment plant. For this purpose, 3, 6, 9, 12 and 15 particles were added to glass tubes containing 4.5 mL of 60 μg MC-LR/L, 60 μg CYN/L and 10 μg ATX-A/L and incubated for 2 h. For the P-Mes particles, the percentage of MC-LR removal improves significantly when particle: volume ratio increases from 1:1500 μL to 1:750 μL, achieving an almost complete (96.95 ± 0.86 %) removal with 6 particles in 4.5 mL (Fig. 5A). However, further increase of the number of particles did not provide significant improvement of MC-LR removal (Fig. 5A). The adsorption of CYN and ATX-A by P-Mes also improves significantly as the particle: volume ratio increases, but only moderate adsorption values can be reached, even at the highest number of particles (Fig. 5A).

For P-MAC particles, the adsorption of CYN increased from 85.15 ± 2.63 % at a 1:1500 μL ratio to nearly complete removal (92.83 ± 1.2 %) at a ratio of 1 particle per 500 μL of water (Fig. 5B). Moreover, the percentage of ATX-A removal with these particles is almost complete with a ratio of 1 particle per 1500 μL, so increasing the particle: volume ratio from initial experimental conditions did not significantly change the adsorption. Finally, the adsorption of MC-LR increases as the number of particles is increased, reaching 73.16 ± 5.77 % at 1 particle per 300 μL of water (Fig. 5B).

3.3.4. Toxin concentration

To test the effect of different toxin concentrations on particle adsorption, three stock solutions containing 180 μg/L of MC-LR, 360 μg/L of CYN or 40 μg/L of ATX-A were prepared, and diluted further to the following concentrations: 180, 150, 120, 90, 60, 30, and 10 μg/L for MC-LR; 360, 300, 240, 180, 120, 60, and 20 μg/L for CYN; and 40, 33.3, 26.6, 20, 13.3, 6.6, and 2.2 μg/L for ATX-A. Then, 3 P-Mes particles were used to evaluate the adsorption at each one of the MC-LR concentrations, while 3 P-MAC were used for CYN and ATX-A, at a particle: volume ratio of 1:1500 μL for 2 h. The data revealed differences in the amount of adsorbed toxin per g of particles, which increases as toxin concentration rises (Fig. 6). At the highest toxin concentrations, P-Mes particles show an adsorption of 16.84 ± 0.83 μg MC-LR/g (Fig. 6A), while P-MAC particles showed an adsorption of 63.67 ± 4.8 μg CYN/g (Fig. 6B) and 7.33 ± 0.65 μg ATX-A/g (Fig. 6C). The adsorption profiles, after exploring fitting with the Langmuir isotherm model, suggest that the particles do not reach saturation at the highest toxin concentrations tested, due to the linear trend observed (Fig. 6), which did not allow for adequate estimation of maximum binding capacity and equilibrium

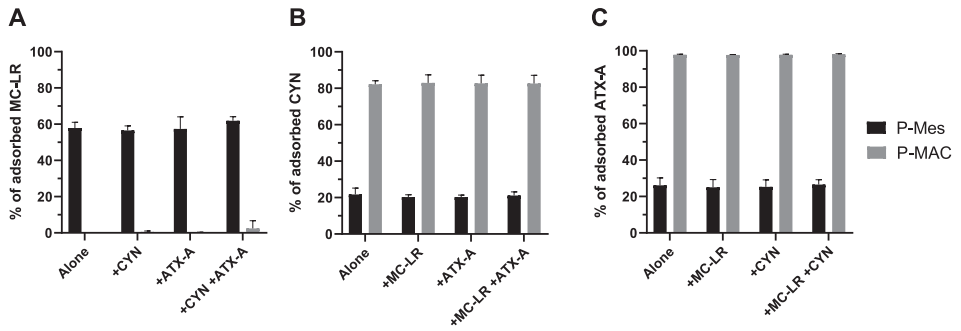

Fig. 2. Adsorption of representative cyanotoxins to P-Mes and P-MAC nanostructured particles when present simultaneously in the same solution. Adsorption of MC-LR (A), CYN (B) and ATX-A (C) alone and in combination with one or two toxins was evaluated after exposure for 2 h to nanostructured composites P-Mes or P-MAC. Experiments were done keeping 3 nanostructured particles in contact with 4.5 mL of a solution of one, two or three toxins, at concentrations of 60 μg/L for MC-LR and CYN and 10 μg/L for ATX-A. The results are reported as percentage of removal calculated after quantification of initial and final concentrations of the toxins. No statistical differences were observed for each toxin in the adsorption separately and in combination with the other toxins (mean ± SD, n = 3).

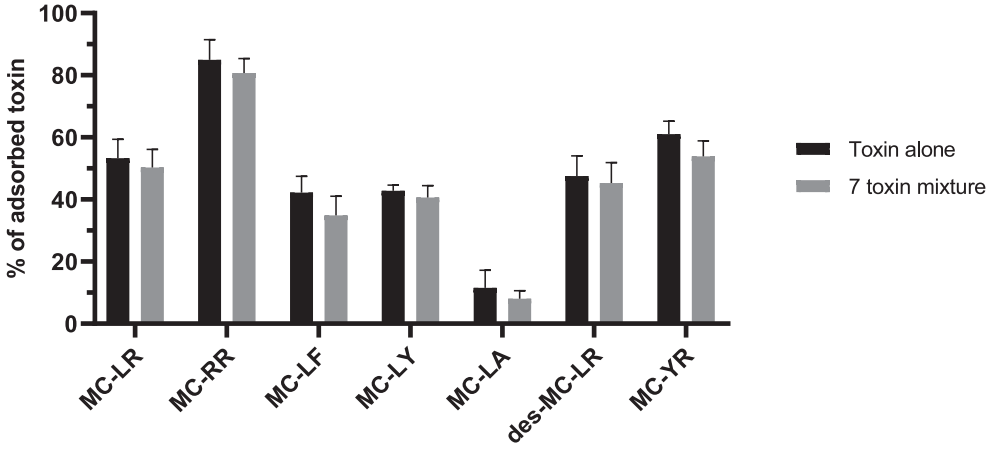

Fig. 3. Adsorption of P-Mes nanostructured particles for 7 MC analogues alone or in combination with other 6 analogues. The experiments were performed by keeping in contact P-Mes particles with solutions of 60 μg/L MC-LR, MC-RR, MC-LF, MC-LY, MC-LA, des-MC-LR and MC-YR and one solution with the 7 toxins at the same concentration (particle-to water volume ratio of 1:1.5 mL). No statistical differences were observed in the adsorption separately and in combination with other toxins (mean ± SD, n = 3).

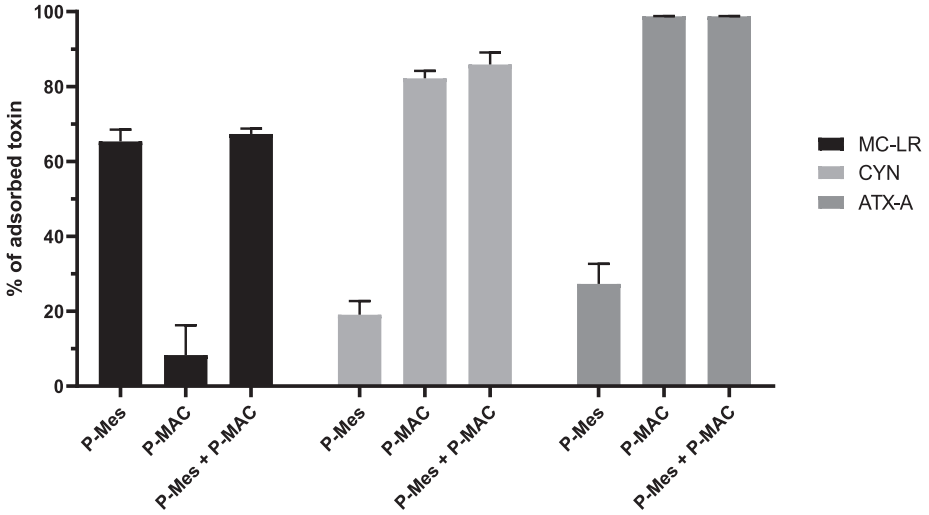

Fig. 4. Adsorption of P-MAC and P-Mes particles alone or in combination for MC-LR, CYN and ATX-A. Experiments were conducted using 3 nanostructured particles in 4.5 mL and, when combination of particles was tested, 3 nanostructured particles of each type were added to 4.5 mL. Initial concentrations of the three toxins in the test solution used were 60 μg/L for MC-LR and CYN and 10 μg/L for ATX-A. No statistical differences were observed between the adsorption of the best particle for each toxin and the combination of both particles (mean ± SD, n = 3).

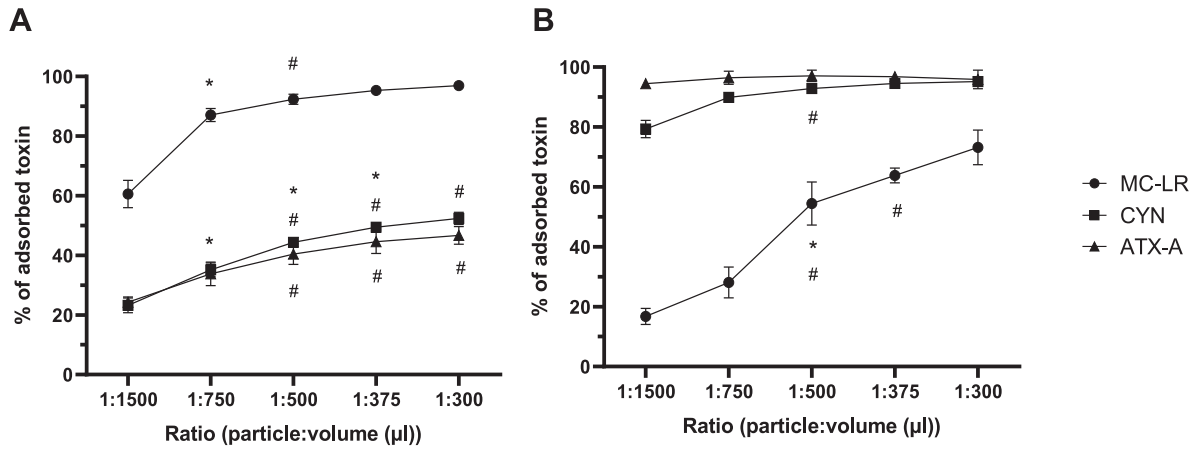

Fig. 5. Effect of the particle: volume ratio on the adsorption of MC-LR, CYN and ATX-A to P-Mes (A) and P-MAC (B) nanostructured particles. A volume of 4.5 mL of toxin solutions at concentrations of 60 µg/L for MC-LR and CYN and 10 µg/L for ATX-A was exposed for 2 h to 3, 6, 9, 12 and 15 particles of P-Mes or P-MAC composites. Percentage of adsorbed toxin was calculated considering pre- and post-exposure toxin concentrations (mean ± SD, n = 3). *: Statistically significant vs the immediately lower particle: volume ratio condition; #: Statistically significant vs the second-lower particle: volume ratio condition ($p < 0.05$).

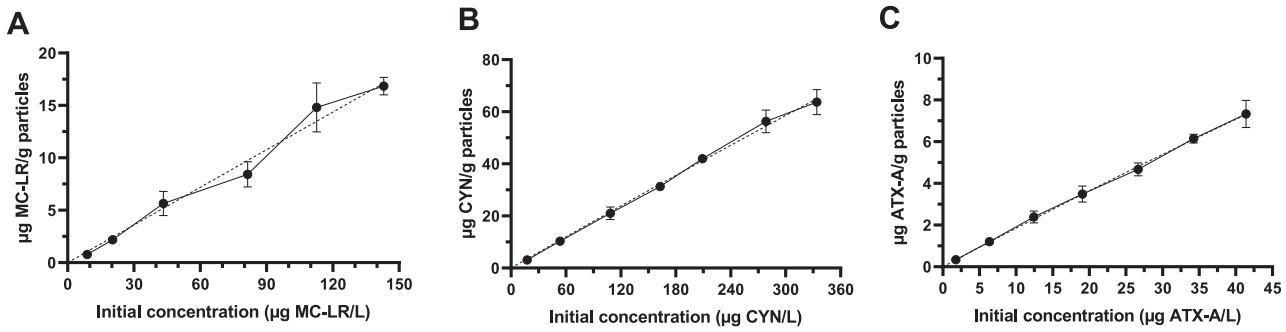

Fig. 6. Effect of different toxin concentrations on MC-LR, CYN and ATX-A adsorption to nanostructured composites. Experiments were performed keeping 3 nanostructured particles in contact with 4.5 mL of a solution of toxin at different concentrations. MC-LR adsorption by P-Mes (A) was tested at concentrations of 180, 150, 120, 90, 60, 30, and 10 µg/L, CYN adsorption by P-MAC (B) at concentrations of 360, 300, 240, 180, 120, 60, and 20 µg/L and ATX-A adsorption by P-MAC (C) at concentrations of 40, 33.3, 26.6, 20, 13.3, 6.6, and 2.2 µg/L. Langmuir model isotherm fit is indicated as a dashed line. For this model R^2 was 0.982 in A, 0.9867 in B and 0.9852 in C (mean ± SD, n = 3).

dissociation constant. Nonetheless, the percentage of toxin adsorbed for each particle/toxin pair (P-Mes/MC-LR, P-MAC/CYN and P-MAC/ATX-A) was similar at all toxin concentrations.

3.4. In vitro toxicity evaluation

3.4.1. Cytotoxicity of P-Mes and P-MAC pulverized particles in SH-SY5Y, CACO-2, HepG2 and CAKI-1 cells

To assess possible toxic effects in extreme physical deterioration conditions, in vitro cytotoxicity of pulverized P-Mes and P-MAC particles was tested in four cell lines: CAKI-1, CACO-2, HepG2 and SH-SY5Y. Dry particles were pulverized using a vibration mill (Retsch MM2) at 50 cycles/s for 1 min. Suspensions of the pulverized particles were prepared in water or medium at concentrations of 125 µg/mL, 250 µg/mL, 500 µg/mL, 1 mg/mL, 2 mg/mL and 4 mg/mL and maintained in constant shaking for 24 h. After centrifugation, the water samples were added to the wells at a concentration of 50 % of final volume. For cell cultures treated with medium exposed to pulverized particles, culture medium was removed previously to the addition of the particle-incubated medium. Cell metabolic activity was measured at 4, 8, 12, 24 and 48 h after treatment using the fluorescent cell viability test AlamarBlue. Controls of water or medium not exposed to the particles were performed in the same conditions. The results showed that there were no significant differences at any time in the raw fluorescence data of treated cells compared to the Milli-Q water or medium controls at any of the concentrations tested. The results are displayed as percentage of viability relative to control, only data for the higher concentration tested (4 mg/mL) are shown (Fig. 7).

3.4.2. Effect of P-Mes and P-MAC particles on trans-epithelial electric resistance of CACO-2 monolayers

CACO-2 cells differentiated for 21 days were treated with water or medium in contact with the P-Mes or P-MAC particles. For water samples a ratio of 1 particle per 1.5 mL of water was used for exposure to nanostructured particles for 2 h and then added to cell medium in contact with the apical membrane of the cell monolayer at a concentration of 50 %. For medium samples a concentration of 4 mg/mL of pulverized particles was incubated for 24 h with culture medium and centrifuged for debris separation; the supernatant was used to replace the culture medium in contact with the apical membrane of the cell monolayer in the hanging insert. A parallel control with Milli-Q water or medium that were not in contact with particles was conducted in the same conditions. *Trans*-epithelial electric resistance of the CACO-2 monolayers was measured before and after treatment and used to calculate resistivity, and the results are presented as a percentage of resistivity relative to the pretreatment (Fig. 8). No significant differences were found in membrane permeability of the cells treated with water or cell culture exposed to the particles compared to the controls.

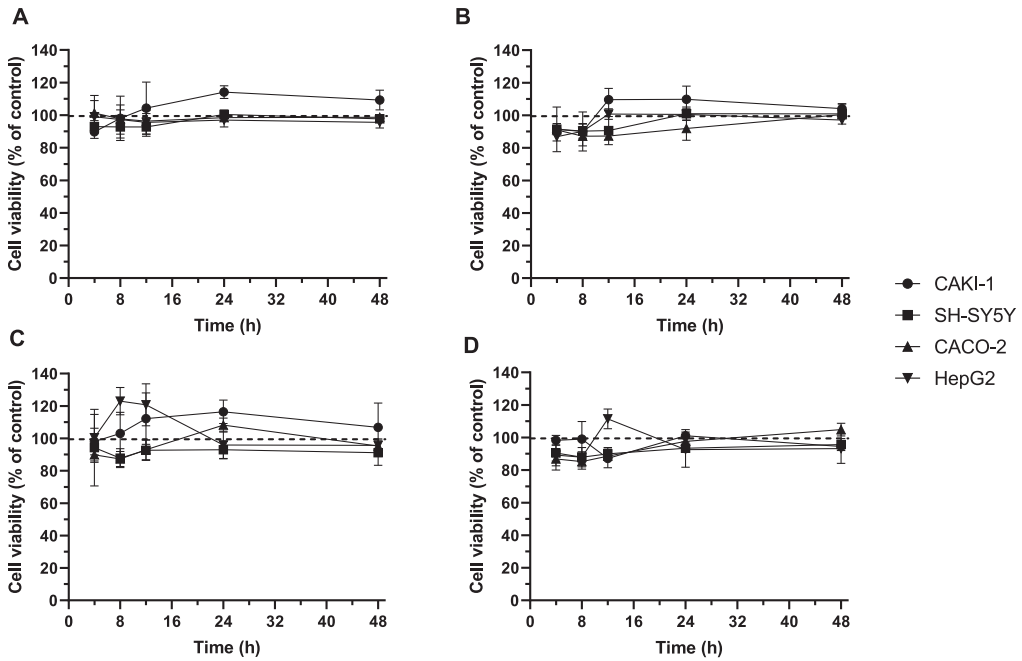

Fig. 7. In vitro toxicity of pulverized nanostructured particles in CAKI-1, SH-SY5Y, CACO-2 and HepG2. P-Mes (A and C) and P-MAC (B and D) pulverized particles were suspended in water (A and B, respectively) or medium (C and D, respectively) at a concentration of 4 mg/mL for 24 h. Pulverized material suspension was then separated by centrifugation. Cells were treated with the supernatant by addition to the culture well of 50 % *v*/v of water or replacement of culture medium with particle-treated medium. AlamarBlue was used as viability reporter and its fluorescence was measured at 4, 8, 12, 24 and 48 h after treatment. Results are expressed as percentage of fluorescence intensity of controls (water or medium not exposed to particles). Each sample was tested in triplicate. Data differences were not statistically significant (mean ± SD, n = 3).

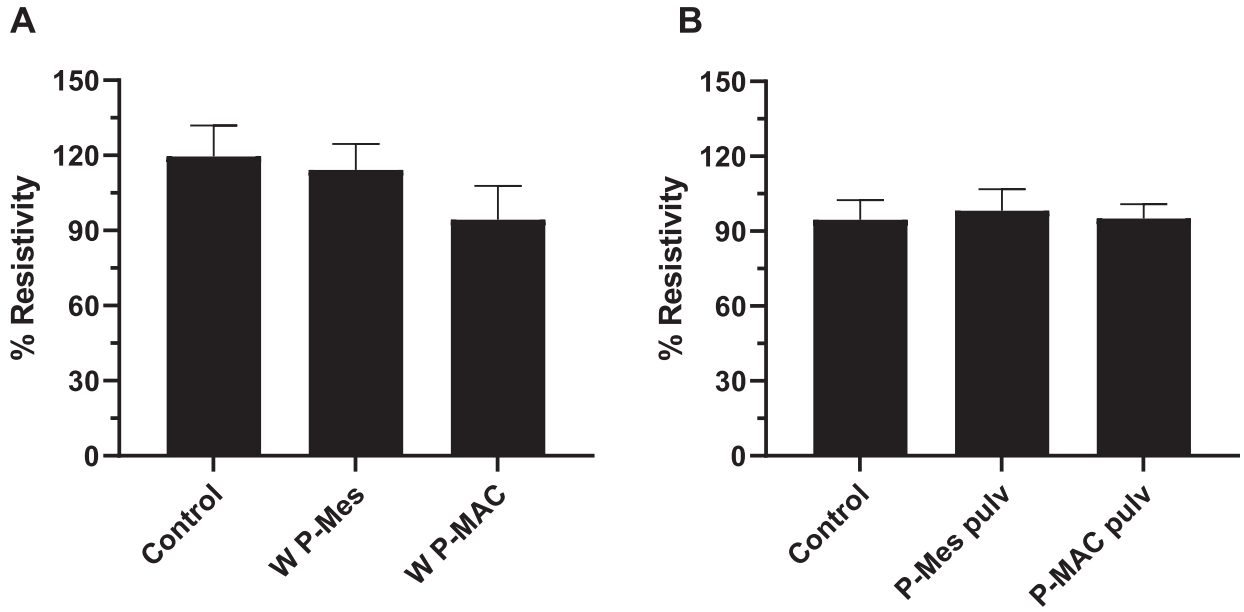

Fig. 8. Effect of P-Mes and P-MAC particles on CACO-2 monolayer permeability. CACO-2 cells were placed in inserts and differentiated into enterocytes for 21 days. (A) Cells were treated with Milli-Q water exposed to nanostructured P-Mes or P-MAC particles at a concentration of 1 particle each 1.5 mL of water for 2 h, by addition to the apical side of the CACO-2 culture at a concentration of 50 % v/v. (B) Cells were treated with medium exposed to pulverized particles at a concentration of 4 mg/mL for 24 h by addition of pulverized particle-treated medium to the apical side of the cell culture after removal of old culture medium. Resistivity was calculated from the trans-epithelial electrical resistance measured before and 24 h after treatment, and the results are presented as a percentage of post-treatment resistivity relative to the pre-treatment value. No statistical differences were found (mean ± SD, n = 3).

4. Discussion

The optimized cyanotoxin removal method presented in this study uses magnetic nanostructured particles with mesoporous carbon (P-Mes) and activated carbon (P-MAC) as adsorbent material. The superparamagnetic properties of the magnetite nanoparticles in the composites ensure dispersion in aqueous suspension that provides adequate contact with toxins in solution, besides efficient particle collection with a magnet. This method allows to remove 10 cyanotoxins from water and is highly efficient removing the most common toxins,

specifically MC-LR, MC-RR, CYN, ATX-A and STX, achieving for these five molecules adsorption rates higher than 84 %. Regarding the adsorption of microcystins by P-Mes, there is significant variability among analogues (MC-RR > MC-LR ≈ MC-YR ≈ des-MC-LR > MC-LF ≈ MC-LY > MC-LA), ranging from MC-RR with an adsorption rate of 85 % to MC-LA with only 13 % adsorption. Although to the extent of our knowledge no previous study about removal of cyanotoxins by adsorption has tested as many analogues; a similar variation was obtained in previous publications with carbon-based adsorbents in the adsorption of MC-RR, MC-LR, MC-YR and MC-LA [31–33]. These adsorption differences may be due to the variable amino acids present in the heptapeptide MC structure. Analogues with hydrophilic amino acids, such as arginine (R), provided better adsorption rates to the particles compared to those with hydrophobic amino acids, such as leucine (L), alanine (A), phenylalanine (F), and tyrosine (Y). These variations in amino acids alter the polarity of the molecule and the electrostatic interactions [34], changing the attraction to the mesoporous carbon.

STX has not been extensively studied for its removal from water, despite being one of the most frequent cyanotoxins detected in freshwater [4]. In this study, almost complete adsorption was achieved by both P-Mes and P-MAC particles, which suggests that pore diameter may not be a significant parameter in STX adsorption, considering the differences in pore size of both materials, since mesoporous carbon pores are mainly between 2 and 50 nm, while micropores smaller than 2 nm are predominant in activated carbon. This high efficacy may be due to electrostatic interactions, as both carbon-based adsorbents have negative charge at the pH of Milli-Q water [35], while STX has a positive charge [36], resulting in attractive forces. Since STX adsorption seems to depend on electrostatic interactions, the adsorption efficiency varies significantly with different pH values of the solution [37]. In general, the percentage of STX removal by P-MAC in Milli-Q water pH (around 5.7) is better than other particles reported, like carbon-based magnetic nanoparticles in a solution of pH 6.99 [25], or chitin particles [38] and oyster-shell-powder particles in solutions of pH 5 and 7 [38]. In the experimental conditions of this study and those previous works, STX would be positively charged, as it would be also the case in natural waters that commonly have a pH lower than 8.2. When the kinetics of STX adsorption to these materials are compared with ATX-A adsorption kinetics to P-MAC [26], the adsorption of STX appears to be slower, in spite of displaying removal rates at 2 h similar to ATX-A. In the case of ATX-A, almost complete adsorption is achieved 60 min after particle addition [26]. This difference might be due to ATX-A being a smaller molecule, which allows it to enter the particle's micropores with less spatial hindrance than STX, as observed in other studies with different molecular weight adsorbates [39].

In this study, homogeneity of particle batches was explored, showing varying levels of adsorption efficiency for MC-LR and ATX-A by P-Mes, and for MC-LR by P-MAC, likely due to slight differences in the fabrication process. However, the adsorption of MC-LR by P-Mes in the low adsorption batch was raised to the same level of the other batch using ethanol washes, suggesting that a pre-conditioning procedure, before toxin removal with these particles, can be used to standardize batch adsorption properties. Notably, the adsorption of CYN by P-MAC particles doubled when pre-conditioned with 75 % acetonitrile or ethanol. It is possible that these organic solvents, ethanol and acetonitrile, act by cleaning the pores of the particles, making them more accessible for toxin adsorption, thus significantly enhancing their capacity to remove toxins. Although acetonitrile may provide a more efficient preconditioning for P-MAC, ethanol pre-treatment provided similar results, and might be regarded also as a suitable option considering costs and uniformity of production processes for both particles. Remarkably, adsorption properties of these composites of magnetite nanoparticles and carbon-based adsorption materials can be significantly improved by pre-treatment with some organic solvents, and to the extent of our knowledge no other studies have reported solvent preconditioning of adsorbent materials to improve performance. Although other procedures aimed at increasing adsorption in carbon-based materials have been explored in other studies, such as functionalization by adding amino groups on the adsorption surface [39], they did not yield an increase of adsorption capacity, despite involving more expensive and complex methods.

The performance of these nanostructured composites regarding toxin removal when several toxin groups or several different analogues are present in water is crucial for water treatment processes. This study revealed that cyanotoxins, whether from the same group or different groups, do not compete for adsorption sites on the particles. This performance feature is a significant advantage because some cyanobacteria, like *Microcystis aeruginosa,* can produce multiple microcystins during proliferation [40]. In the case of a *M. aeruginosa* bloom, a single particle type, P-Mes, can eliminate several analogues, simplifying the removal process. Moreover, a single species of cyanobacteria can produce cyanotoxins from different groups, such as *Anabaena flos-aquae*, which produces ATX-A and four MC analogues [41]. When more than one group is implicated, and one of them is MCs, the combination of P-Mes and P-MAC particles could be the best alternative, as it has been shown that their use simultaneously does not compromise their adsorption capacities. However, this combination does not enhance the overall efficiency of adsorption, possibly because P-MAC is not an effective adsorbent for MC-LR, nor is P-Mes for CYN. In the case of ATX-A, although P-Mes can moderately adsorb the toxin, it is not possible to evaluate any increased adsorption in the conditions tested since P-MAC alone is already capable of nearly complete toxin removal.

For practical application in water treatment plants, in addition to toxin concentration in the solution, consideration of the optimal particle-to-volume ratio is essential, which will lead to adequate proportions of initial toxin amount per particle. In this study, near-complete adsorption of MC-LR by P-Mes particles was achieved with a ratio of 1 particle per 750 μL of water (average of 8 mg of adsorbent composites/mL) for 60 μg/L MC-LR (45 ng of MC-LR in the initial solution per particle). Conversely, P-MAC particles effectively adsorbed CYN and ATX-A at a lower ratio of 1 particle per 1.5 mL (average of 4 mg of adsorbent composites/mL) for 60 μg/L CYN or 10 μg/L ATX-A (90 ng CYN and 15 ng ATX-A per particle). These ratios are an improvement over previous studies that also use large-sized activated carbon and mesoporous carbon particles, which required a ratio of 1 particle per 200 μL of water to obtain a removal percentage of 90 % MC-LR and 70 % for CYN and ATX-A [32]. When compared to nanoparticles, the ratios are typically reported in mg/mL rather than by particle count [21,42]. These data are not comparable with this study due to the incorporation of magnetite to the particles, which results in higher weight data, but this addition provides significant practical benefits, such as facilitating easier particle recovery and reuse.

Notably, no saturation of P-Mes or P-MAC particles was observed within the range of toxin concentrations typically found in natural waters, usually between 0.1 and 20 μg/L [43], highlighting their potential applications in the water treatment process. In fact, the amount of toxin adsorbed to P-Mes and P-MAC increases proportionally to initial toxin concentrations, likely due to the availability of non-specific binding sites that have not yet reached saturation. This characteristic ensures a constant rate of toxin removal in terms of percentage of initial concentration at toxin levels of most cyanobacterial blooms. While previous studies on MC-LR have reported saturation at higher toxin concentrations [20,44,45], the concentrations used in those experiments exceeded the levels typically encountered in natural environments.

Although the in vitro toxicity of water in contact with P-Mes and P-MAC particles has been studied in previous publications [26], a more comprehensive study on the potential toxicity of these nanostructured particles has been conducted in conditions of extreme physical damage of the particles to further test material safety. The results indicate that pulverized particles do not release materials into the water or medium that are toxic to four human cell lines, because metabolic activity of these cell types and integrity of transepithelial barrier in CACO-2 cells

was not altered. These cell models were tested because they represent tissues most likely to be exposed to any compound administered orally or to be particularly affected in cases where the intestinal barrier is compromised. These preliminary results showing lack of in vitro toxicity should be complemented in the future with in vivo acute, sub-acute and chronic toxicity studies before scaling this technology to a drinking water treatment plant. In addition, long-term particle stability during storage should be evaluated before future practical implementation to ensure adequate waste treatment for environmental risk mitigation.

5. Conclusions

Magnetic nanostructured composites of magnetite nanoparticles and two adsorbent materials, mesoporous carbon (P-Mes) or activated carbon (P-MAC), provide efficient cyanotoxin removal from aqueous solution. The results demonstrate the effectiveness of P-Mes and P-MAC in adsorbing a range of free cyanotoxins from water, including MCs, CYN, ATX-A, and STX, after 2 h of exposure to the composites. P-MAC removal of CYN, ATX-A, and STX is higher than 85 %, and P-Mes adsorbs at least 6 MCs, with efficiencies close to 60 % and 80 % for the most common analogues MC-LR and MC-RR respectively. Interestingly, we show that toxin adsorption by P-Mes and P-MAC particles is increased by preconditioning of the composites with organic solvents. Furthermore, optimizing the particle-to-volume ratio can be used to improve the method efficiency during its application in drinking water treatment plants. Finally, both types of particles can be effectively used to remove multiple toxins simultaneously, as no competition for adsorption among these compounds or inhibition when combining the different materials occurs. Notably, no toxicity of the materials was observed in conditions of physical deterioration of the particles, nor was there any alteration of the intestinal barrier in the in vitro assays, suggesting the safety of these particles for future use.

CRediT authorship contribution statement

Alejandro Cao: Writing – original draft, Methodology, Investigation, Formal analysis. **Natalia Vilariño:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Lisandra de Castro Alves:** Methodology, Investigation, Formal analysis. **Yolanda Piñeiro:** Writing – review & editing, Supervision, Formal analysis. **Jose Rivas:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Ana M. Botana:** Methodology, Formal analysis. **Cristina Carrera:** Methodology, Formal analysis. **María J. Sainz:** Methodology, Formal analysis. **Luis Rodríguez-Santos:** Methodology. **M. Carmen Louzao:** Formal analysis. **Luis M. Botana:** Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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