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CNTs coated charcoal as a hybrid composite material: Adsorption of fluoxetine probed by zebrafish embryos and its potential for environmental remediation.

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HIGHLIGHTS

- HMNC and AC are practically non-toxic to zebrafish embryos: LC_{50} (96 h) $\geq$ 1000 mg.L^{-1}.
- HMNC adsorbs fluoxetine molecules more effectively than AC.
- Fluoxetine interacts with HMNC and is less available to early embryo development stages.
- Embryos exposed to HMNC and unexposed embryos behave similarly.

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ABSTRACT

Although traditional water treatment systems can remove various substances from wastewater, these conventional systems fail to remove many chemical molecules that pose potential ecological and health risks. Carbon nanotubes (CNTs) appear attractive to adsorption of many substances, but CNTs adsorbed with toxic substances becomes a nanocomposite still more toxic. Here, we employ zebrafish embryos as biosensor to examine how a hybrid micro/nanostructured carbonaceous material (HMNC) derived from a combination of activated carbon (AC) with hydrophilic carbon nanotubes (CNTs) can remediate wastewater contaminated with the pharmaceutical fluoxetine hydrochloride (FLX). AC and HMNC are practically non-toxic to zebrafish embryos (LC_{50} > 1000 mg.L^{-1}). HMNC addition to culture medium containing FLX significantly reduces sublethal effects and lethality. Interaction between FLX and HMNC involves chemical adsorption such that embryo co-exposure to HMNC adsorbed with FLX in the range of concentrations evaluated herein does not elicit any behavioral changes in zebrafish.

1. Introduction

Inefficient fluoxetine hydrochloride (FLX) removal by conventional water treatment and the consequent broad distribution of this drug in the environment (Aus der Beek et al., 2015) make the development of more efficient water treatment methods and of materials that can be incorporated into water treatment plants an urgent matter.

Activated carbon (AC) can remove many pharmaceutically active compounds from water, so it plays an important role in remediating water containing emerging contaminants. Different kinds of AC exist depending on their physicochemical properties (e.g., particle size, pore size distribution, surface area, and bulk density). The functional groups distribution on the AC surface determines how AC interacts with contaminants. Adsorption is controlled by van der

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Waals interactions between AC and non-polar contaminants or electrostatic interactions between the charged AC surface and polar contaminants. However, AC has disadvantages, including difficult reactivation and need for longer contact with the contaminant for adsorption to occur (Yoon et al., 2008; Mccreary and Snoeyink, 2014; El Gamal et al., 2018). Thus, carbon nanomaterials have been studied as alternative to improve micropollutant removal in water (Cho et al., 2011; Sotelo et al., 2012).

Several carbon nanomaterials have been studied for micropollutant removal (Cho et al., 2011; Sotelo et al., 2012; Ji et al., 2010). In this context, carbon nanotubes (CNTs) have been claimed as one of the most promising remediation materials for their large specific surface area, high porosity, high selectivity, numerous adsorption sites, short intraparticle diffusion distance, and tunable pore size, not to mention that they can engage in desirable interactions with countless contaminants and can be produced on a large scale. In spite of CNTs can be potentially applied in water remediation, their similarities with asbestos and their possible cyto- and genotoxic effects (Franchi et al., 2012) have raise concerns. Various studies have shown that CNTs impact terrestrial and aquatic organisms negatively (Lam et al., 2004; Warheit et al., 2004; Donaldson et al., 2006; Templeton et al., 2006; Roberts et al., 2007; Smith et al., 2007). In aquatic environment the CNTs when combined with toxic adsorbents become indeed a harmful element, and this has been recently observed as well as to case of fluoxetine combined with MWCNTs (Yan et al., 2018). Furthermore, there exist additional drawbacks concerning the practical usage of particulate CNTs in water treatment. First, it is hard to obtain hydrophilic CNTs at low costs. Second, using renewable resources to obtain hydrophilic CNTs is difficult because raw CNTs are predominantly hydrophobic. Finally, ensuring that all the nanomaterial will be well exposed to contaminants while avoiding the deleterious CNT-toxic adsorbent release into the aquatic environment is a challenging task.

To overcome the challenges associated with effective commercial use of CNTs in water treatment, here we propose the use of a hybrid micro/nanostructured material (HMNC) consisting of granular active charcoal or active carbon (AC) covered with hydrophilic CNTs grown directly on the AC surface. HMNCs are composites that allow the physicochemical properties of CNTs to be explored as a result of the amount and type of constituents and of the electronic interactions between them. In this kind of hybrid composite material, CNT coating bind to the surface of a dense and micrometric material and can be easily captured by masks, filter paper, etc., which is not possible with free CNTs (a low-density particulate material). HMNCs can be composed of many kinds of micrometric matrixes such as carbon fiber non-woven, charcoal, and bone charcoal and have been little studied in the context of water treatment (Gonçales et al., 2011; Zhang et al., 2009).

We then use zebrafish embryos as biosensor to evaluate how AC or HMNC interacts with FLX. Zebrafish is a small teleost (3–4 cm) belonging to the freshwater fish family Cyprinidae. This fish species is often employed as a model to assess the ecotoxicity of metals, pesticides, pharmaceuticals, and other substances (Kanungo et al., 2014) because the fish are small and undergo rapid external development. Several studies have suggested that acute toxicity measured by using zebrafish embryos (Fish Embryo Toxicity (FET) test –– OECD n. 236) provides similar sensitivity as compared to the adult fish (Lammer et al., 2009). The FET test offers advantages: it requires small volumes of test solutions, consequently generating less waste; the species used in the test are easy to obtain within a short period; and mortality as well as alterations in embryo development can be easily measured (OECD, 2013). To date, zebrafish have not been used as biosensor to check HMNC toxicity to non-target species, and the use of HMNC as adsorbent of psychotropic drugs has not been evaluated to verify whether these composites can be considered as a novel material for application in water treatment and environmental remediation.

2. Materials and methods

2.1. Fluoxetine and activated carbon

Fluoxetine was purchased from C&C pharmaceutical industry (CAS number: 54910-89-3, empirical formula $C_{17}H_{18}F_3NO$). Activated carbon (AC) derived from carbonization of eucalyptus was provided by AlphaCarbo Industrial (particle size = 325 mesh).

2.2. Hybrid micro/nanostructured carbonaceous material

The composite material studied in the present work was synthetized as described previously in detail in Matsubara et al. (2016) and Rosolen et al. (2006). AC was used as a micrometric carbonaceous material. Briefly, AC was submitted to a chemical vapor deposition (CVD) method; alcohol and Co/Mn particles (1:1) were employed as carbon source and catalyst, respectively, to obtain AC covered with CNTs, referred to as hybrid micro/nanostructured carbonaceous material (HMNC) hereafter.

2.3. Samples characterization

The surface charge (zeta potential) was determined by DLS experiments using the Zetasizer Nano ZS apparatus. Samples morphology was assessed by Scanning Electron Microscopy (SEM) using a Zeiss-EVO 50 scanning electron microscope. Energy-dispersive X-ray spectroscopy (EDS) was carried out with the IXRF Systems 500 digital processing for compositional characterization. Specific surface area (SSA) was determined by using the Brunauer, Emmett, Teller (BET) method at 77K and the Quantachrome NOVA 1200 equipment (with N_2). Before the BET experiments, the samples were dried at 150 °C and under reduced pressure for 3 h. The FTIR experiments were performed using a Bruker spectrometer (model Vertex 70). The spectra were recorded using potassium bromide (KBr) pressed pellet containing ~1% w/w of the investigated sample. The measurements were averaged over 96 scans, which were taken at a resolution of 4 cm^{-1} from 400 to 4000 cm^{-1}. The background signal was averaged over 96 scans before each measurement.

2.4. Zebrafish (Danio rerio) embryo culture

This research project was approved by the Ethics Committee on Animal Research of the Institute of Biological Science of the University of Brasilia under protocol number n. 100226/2014.

Zebrafishes were raised in an aquatic facility (ZebTec - Tecniplast, Italy) with a photoperiod cycle of 12:12 h (light:dark) in aquariums under reverse osmosis and activated carbon filtered water. The water parameters were strictly controlled: temperature was maintained at 27.0 ± 1 °C, conductivity at 650 ± 100 μS/cm, pH at 7.0 ± 0.5 and dissolved oxygen ≥95% saturation. These conditions were maintained in all the performed tests. Zebrafish eggs were collected immediately after natural mating, rinsed in water, and checked under a stereomicroscope (Stereoscopic Zoom Microscope – Stemi 2000, Zeiss, Germany). The unfertilized eggs and those showing cleavage irregularities or injuries were discarded.

2.5. Fish embryo toxicity (FET) test

Fish embryo toxicity test was based on the OECD guideline Protocol 236 “Fish Embryo Toxicity” (FET) test (Braunbeck et al., 2014, OECD, 2013). Zebrafish embryos were exposed to different

treatments (single and mixture exposure) prepared by successive dilutions of a stock solution. The test was performed using 60 eggs per treatment, divided in 3 replicates, selected and distributed in 24-well microplates in the climate chamber (SL-24 Solab Científica, Brazil), 20 wells were filled up with 2 mL of the test solution and four wells with water (internal plate control, as required in the OECD guideline). The test was initiated immediately after fertilization, and it was continued for 96 h. Embryos and larvae were observed daily under a stereomicroscope. Developmental parameters were evaluated in embryos over the test period, using a magnification of × 70 for eggs and × 40 for hatched embryos. Before hatching, the following parameters were evaluated: egg coagulation, otolith formation, general delay in development, eye and body pigmentation, somite formation, heartbeat, oedemas, detachment of the tail-bud from the yolk sac, yolk sac absorption and hatching. After hatching, spine malformation and posture (embryos side-lying in the bottom of the microplate well after mechanical stimulus = behavioral changes) were also evaluated. All parameters were assessed and quantified as observed or not observed.

2.5.1. Single and mixture exposure

Zebrafish embryos were exposed to four different concentrations of AC or HMNC (0, 10, 100, and 1000 mg.L^{-1}, in the absence of FLX – pilot test). The FLX toxicity test was performed at the following five different concentrations: 0, 3.19, 4.33, 7.59 and 18.46 mg.L^{-1} (pilot test). After the single toxicity tests (pilot test), the blend bioassays comprised three groups. Group 1 (or FLX Group) consisted of FLX-exposed embryos (0, 3.19, 4.33, 7.59 and 18.46 mg.L^{-1}). For groups 2 and 3 (FLX@AC and FLX@HMNC, respectively), stock solutions of folded and mixed concentrations were prepared in a ratio of 1:1, namely: 0; 6.38; 8.66; 15.18 and 36.92 mg.L^{-1} of FLX and 20; 200; 2000 mg.L^{-1} of AC (or HMNC). After mixed FLX and AC (or HMNC), the mixture concentration will be half of initial. Thus, the final concentrations of the mixtures are: 0, 3.19, 4.33, 7.59 and 18.46 mg.L^{-1} of FLX, and 10, 100 and 1000 mg.L^{-1} of AC (or HMNC). Fig. 1 illustrates the experimental tests with negative control (0 mg.L^{-1}), control of carbonaceous materials (10, 100 and 1000 mg.L^{-1}), single toxicity of the FLX group and their respective mixtures (FLX@AC and FLX@HMNC). The test was performed using 60 eggs per treatment, divided in 3 replicates (20 eggs per replicate and four wells with water (internal plate control, as required in the OECD guideline). The same parameters described in the previous section were analyzed and quantified as observed or not observed.

2.6. High performance liquid chromatography analysis

FLX stock solutions at 2, 10, 20, 30, 40, 50, 80, and 100 mg.L^{-1} concentrations were prepared in the same solvent proportion as the mobile phase and diluted to half of the initial concentration using AC or HMNC 2000 mg.L^{-1} solution to evaluate AC or HMNC saturation. The final concentrations were 1; 5; 10; 15; 20; 25; 40 and 50 mg.L^{-1} of FLX and 1000 mg.L^{-1} of AC or HMNC. Analyses were performed in triplicate; 1 mL was used per replicate at 26 ± 1 °C. The final solutions were kept under stirring, light, and at room temperature for 20 min. Then, they were filtered through a syringe-driven Millex filter (0.22 μm, 13-mm pore) and analyzed by HPLC.

To determine FLX remaining in the medium, the method adapted from Sabbioni et al., (2004) was used. The standard curve was obtained by using a Shimadzu-Prominence high performance liquid chromatograph comprising an online degasser (DGU 20A5 model), a solvent delivery unit (LC-20AT model), an auto sample injector (SIL-20 AHT model), a column oven (20A), an UV-VIS detector (SPD-20A model), and a controller (CBM-20A). The column consisted of C-18-ODS (M) reverse phase CLC (150 mm × 4.6 mm and 5-μm particle size). To construct the standard curve, FLX solutions at concentrations ranging between 1 and 50 mg.L^{-1} were prepared in 35:65 (v/v) phosphate buffer pH 3.0 and acetonitrile. The isocratic method was used. The mobile phase consisted of 35:65 (v/v) phosphate buffer pH 3.0 and acetonitrile at a flow rate of 1 mL min^{-1}. The detection wavelength was set at 228 nm, and the injection volume was 20 μL. The oven temperature was maintained

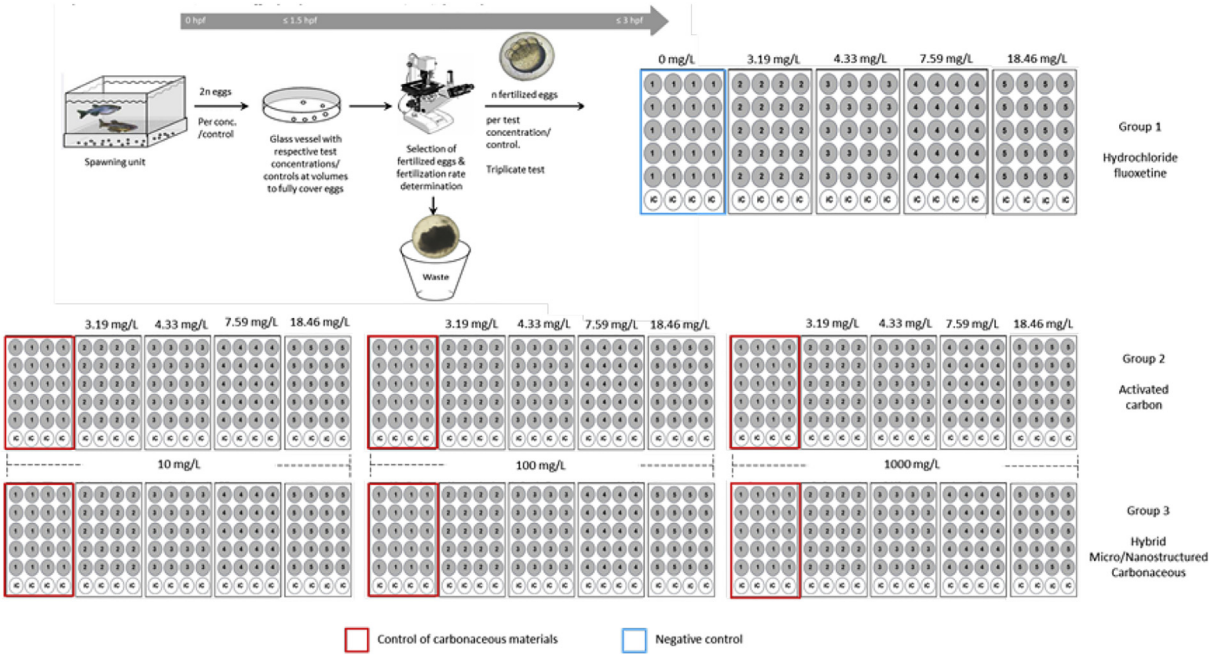

Fig. 1. Experimental embryo toxicity tests, adapted from OECD protocol no. 236.1–5: concentrations of the tested substances; IC: internal control. Group 1: Fluoxetine (FLX); Group 2: Activated Carbon + Fluoxetine (FLX@AC); and Group 3: Hybrid Micro/Nanostructured Carbonaceous Material + Fluoxetine (FLX@HMNC).

at 30 °C. Data were collected and processed with the LC solution software (Shimadzu, Tokyo, Japan).

2.7. Statistical analysis

The effective concentrations (LC_{50} and EC_{50}) to mortality and to behavior change (loss of equilibrium) were calculated using a two-parameter Logistic model. All analyses were performed using the Rstudio statistical package (R Core Team (2018)).

3. Results and discussion

3.1. Morphology of AC and HMNC

AC is a cheap adsorbent extensively studied in the literature and employed in water treatment, and for this reason it is the material chosen to be combined with CNTs. Nevertheless, it is also particularly interesting to preparation of HMNC because its surface is excellent to the incorporation of catalyst used to the CNT growth. Carbonaceous materials, as AC are also attractive to HMNC synthesis since they do not react with catalyst during the CVD methodology used to prepare them Fig. 2 shows SEM pictures of AC with average particle size of 500 μm, whereas Fig. 3 reveals that CNTs cover all the surface of the AC micrometric particles, producing the HMNC. EDS compositional analysis (not shown here) demonstrates that carbon and oxygen predominate in both AC and HMNC, in agreement with the literature (Bansal and Goyal, 2005). AC and HMNC SSA values are 329.4 and 266.0 $m^2 g^{-1}$, respectively, as determined from BET N_2 isotherms. Although CNTs are well known for their large surface area, the lower HMNC SSA as compared to AC is due to blockade of AC pores by CNTs. It is worth to remember that the AC surface is composes by large cavities or holes and small particles of charcoal (inserted in Fig. 2). During the process of the Mn/Co catalyst incorporation on the AC's surface (to CNTs' growth synthesis) these small particles are probably removed. The Mn/Co catalyst precursor salt is expected to penetrate in all large cavities as well as in a fraction of AC's mesopores and micropores too. This could explain the decrease of specific surface area after the AC surface coating with CNTs. In the HMNC, CNTs are found in whole surface of HMNC and inside of the cavities.

DLS measurements allowed determining zeta potential for the carbonaceous nanostructures before and after adsorption with fluoxetine. The values of potential for CA and HMNC pure are $\xi = -30.4 \pm 0.2$ mV and $\xi = -14.3 \pm 0.2$ mV, respectively. After adsorption, these values increased to $\xi = 9.99 \pm 0.2$ mV and $\xi = 16.8 \pm 0.2$ mV, respectively. The positive results of the ξ values indicate the carbonaceous nanostructures were loaded successfully and suggest that fluoxetine adsorption on CNT is probably chemical. Adsorption of FLX on single-walled carbon nanotubes has been studied by simulation and suggests that there exist a charge transfer between FLX and CNTs (Shahabi and Tavakol, 2017). For the case of MWCNTs is still not clear the presence of eventual charge transfer between the MWCNTs and FLX. In supplementary materials is show the he FTIR spectra of free FLX and of FLX adsorbed onto the nanostructure's AC and HMNC (Fig. S1a, ii and iv) do not reveal any significant changes in the FLX vibrational energies. On the other hand, the shifts in the aromatic ring vibration modes and the unchanged behavior of the CF_3 and C-O-C modes suggest that FLX adsorbs onto the carbonaceous nanostructures through π-π interaction. Similar results are found in the literature. The hypothesis that considers π-π interaction agrees with the changes in the AC and HMNC zeta potential values from negative (before FLX adsorption) to positive (after FLX adsorption).

3.2. Biological tests and HPLC analysis

Zebrafish have been widely used in ecotoxicological assessments. In the present study, the embryos present normal development in control medium as well as in carbonaceous adsorbent materials (AC or HMNC), which agrees with previous studies described by Kimmel et al. (1995). The maximum mortality is 3% for the entire test period. We were unable to determine LC_{50} or EC_{50} (sublethal parameters) for HMNC because they are above the highest concentration tested herein (1000 $mg.L^{-1}$).

Literature studies have focused on showing how carbonaceous nanostructures affect non-target species of different trophic levels. However, most of these studies, especially studies on vertebrates, report sublethal effects, generating a knowledge gap about lethality. Additionally, Lovern and Klaper, (2006) pointed out that nanomaterial toxicity depends on the methodology that was used to manufacture them. Therefore, establishing a toxicity pattern is difficult Yu et al. (2016). Zhu et al. (2007) evaluated the toxicity of a carbonaceous nanostructure, C_{60} (fullerene), to zebrafish embryos and observed that the nanomaterial is not toxic at concentrations of up to 50 $mg.L^{-1}$, but the authors were not able to calculate LC_{50} or EC_{50}. Nevertheless, when they evaluated C_n (other fullerene forms), they observed effects on hatching, embryo development, and mortality. Moreover, Cherukuri et al. (2006) assessed the toxicity of CNTs modified with Pluronic to rabbits (intravenous administration), but they did not identify sublethal effects. In contrast, Ye et al. (2009) verified cellular death, morphological changes, ROS, and expression of interleukin-8 (IL-8) genes when they investigated evaluating CNTs dispersed by Pluronic F_{68}. Here, the absence of lethal or sublethal effects on zebrafish embryos shows that AC and HMNC are not toxic to the fish. In our previous study, MWCNTs were tested in zebrafish for cyto- or genotoxicity. The lack of cyto-

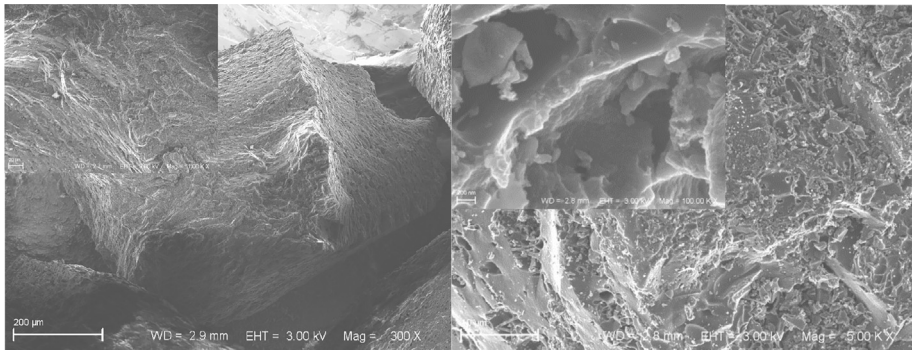

Fig. 2. SEM images of Activated Carbon (AC) at different magnifications.

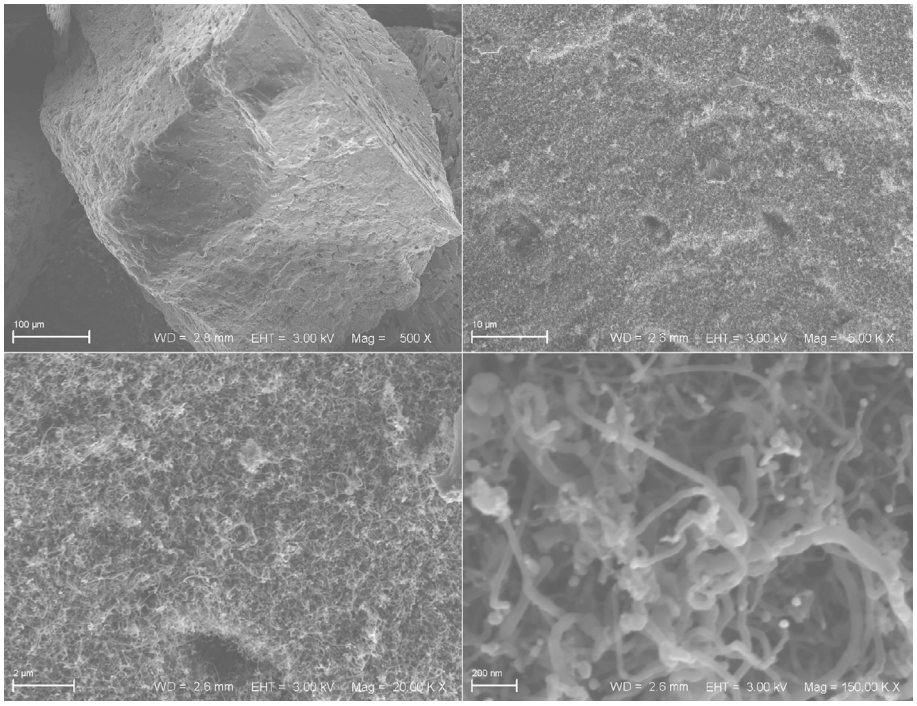

Fig. 3. SEM images of Hybrid Micro/Nanostructured Carbonaceous Material (HMNC) at different magnifications.

genotoxicity and reactivity encouraged more refined studies with purposes for using this nanomaterial in remediation processes Souza Filho et al. (2014).

The lethality results obtained with control FLX (LC_{50} 96 h = 6.32 mg.L^{-1}) can be compared to literature studies. For example, Nakamura et al. (2008) evaluated FLX effects on *Oryzias latipes* fish and determined LC_{50} 96 h = 5.5 mg.L^{-1}. Brooks et al. (2003) assessed the FLX environmental risk to selected benthic and pelagic toxicity test organisms, to obtain LC_{50} 168 h = 0.89 mg.L^{-1} for *Pimephales promelas* fish. Henry and Black (2008) achieved LC_{50} 168 h = 0.56 mg.L^{-1} for the adverse FLX effects on *Gambusia affinis* fish. Alsop and Wood (2013) conducted a study on zebrafish, to find lower acute FLX toxicity: LC_{50} 96 h = 0.25 mg.L^{-1}.

Table 1 lists the LC_{50} values for the positive control (FLX) and for FLX in the presence of different concentrations of carbonaceous adsorbents (AC or HMNC).

For AC or HMNC at 10 mg.L^{-1}, LC_{50} is around 34% higher for HMNC. In the case of AC or HMNC at 100 mg.L^{-1}, LC_{50} is approximately 6.5% higher for AC. A tenfold increase in AC concentration raises LC_{50} by 38.6%, whereas a tenfold increase in HMNC concentration raises LC_{50} by only 1.1%. Therefore, a lower HMNC concentration provides optimal results in terms of LC_{50}. In other words, HMNC 10 mg.L^{-1} is equivalent to AC 100 mg.L^{-1}, which represents an advantage of HMNC over AC when it comes to minimizing zebrafish embryo mortality.

FLX@AC 1000 mg.L^{-1} and FLX@HMNC 1000 mg.L^{-1} had *no mortality effect*, so we were no able to calculate LC_{50}. Fig. 4 shows an overview of the embryo toxicological results.

Although LC_{50} is higher for AC 100 mg.L^{-1} as compared to HMNC 100 mg.L^{-1}, HMNC does not elicit any behavioral changes in the organism, whereas mortality in the presence of FLX at 4.33, 7.59, and 18.46 mg.L^{-1} is 5%, 10%, and 100%, respectively.

Serotonin (5-HT) is one of the most important and ubiquitous neurotransmitters in the animal kingdom (Azmitia, 1999). In aquatic organisms, the serotonergic system (the FLX action site) plays a fundamental role in their behavior: social, food demand, and motor responses (Lillesaar et al., 2007; Mennigen et al., 2010). Studies have shown that aquatic organisms exposed to FLX concentrations display altered behavior (Airhart et al., 2007; Prieto, 2012). We evaluated behavioral changes (equilibrium) in hatched embryos after 96 h of exposure to different samples at different concentrations. Fig. 5 shows that approximately 80% of the organisms exposed to 4.33 mg.L^{-1} of FLX are altered. Almost all the organisms exposed to FLX 7.59 mg.L^{-1} died (only n = 2 live organisms in 60 - these data was not showed in Fig. 5). In this case, the only two remaining hatching embryos present behavioral changes, which means 100% behavior change. We were not able to measure behavioral changes in the organisms exposed to FLX 18.46 mg.L^{-1}, because they were all dead at 96 h.

Less than 20% and 2% of the organisms exposed to FLX 4.33 mg.L^{-1}@AC 10 mg.L^{-1} and FLX 4.33 mg.L^{-1}@AC 100 mg.L^{-1}, respectively, have altered behavior. These data reveals a dose-dependent response, as it was detected less behavioral changes when AC concentration increases.

To FLX 7.59 mg.L^{-1}@AC 10 mg.L^{-1}, AC maintains approximately 50% of living organisms, but all of them had changes in the balance. Besides of, it was observed behavioral changes amount to 10% in

Table 1
Lethal concentrations in mg.L^{-1} (±standard error) of Fluoxetine (FLX) and mixtures of Fluoxetine + Hybrid Micro/Nanostructured Carbonaceous Material (FLX@HMNC) and Fluoxetine + Activated Carbon (FLX@AC) used in the zebrafish embryo toxicity tests.

Treatment	LC_{50} (96 h)	Model (R^2)
FLX	5.20 ± 0.19	Logistic - two parameters (0.98)
FLX@AC 10 mg.L^{-1}	7.54 ± 0.31	Logistic - two parameters (0.99)
FLX@AC 100 mg.L^{-1}	11.25 ± 0.71	Logistic - two parameters (0.98)
FLX@HMNC + 10 mg.L^{-1}	10.08 ± 0.60	Logistic - two parameters (0.98)
FLX@HMNC + 100 mg.L^{-1}	10.52 ± 0.65	Logistic - two parameters (0.99)

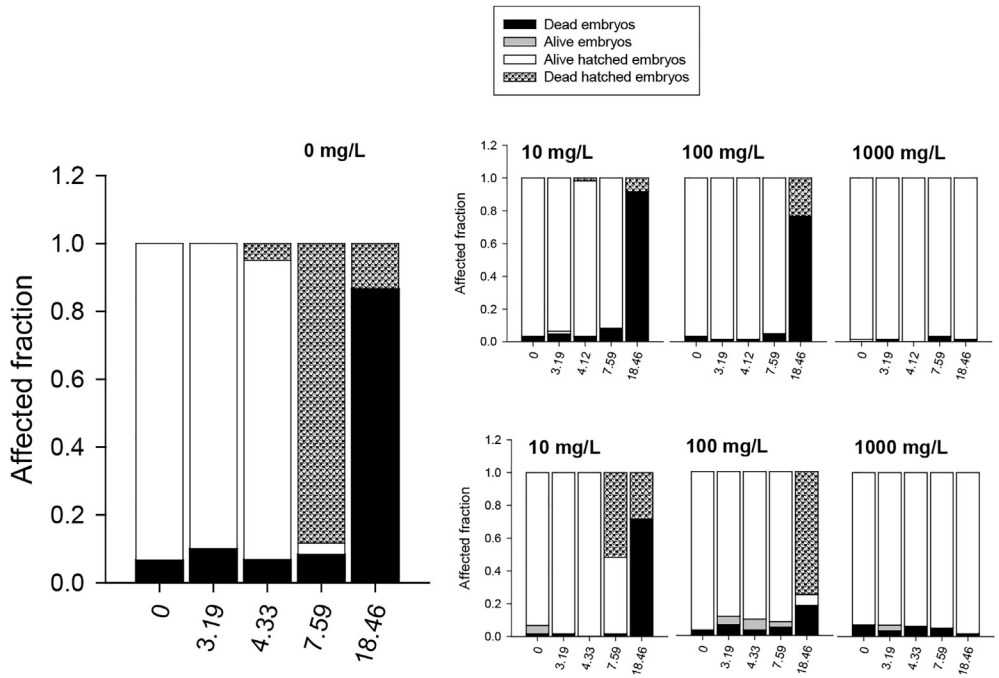

Fig. 4. Overview of the embryotoxicity studies after 96-h exposure to different treatments. Letter "a" represents the Fluoxetine (FLX) treatment. Letters "b", "c", and "d" corresponds to the different treatments with Fluoxetine + Hybrid Micro/Nanostructured Carbonaceous Material (FLX@HMNC), whereas letters "e", "f", and "g" refer to the different treatments with Fluoxetine + Activated Carbon (FLX@AC).

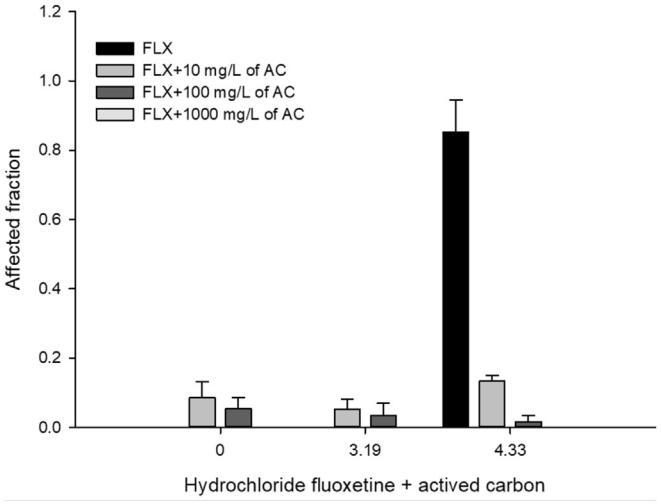

Fig. 5. Bar graph (±standard error) of the behavioral changes at 96 h of exposure to Fluoxetine (FLX) and FLX@Activated Carbon (FLX@AC).

FLX 7.59 mg.L^{-1}@AC 100 mg.L^{-1}. In addition, less than 7% of the organisms (n = 4) are alive in FLX 18.46 mg.L^{-1}@AC 100 mg.L^{-1}, and all of them had changes in equilibrium. Table 2 shows the effect concentrations (EC_{50}) for organisms exposed to FLX and FLX@10 mg.L^{-1} of AC.

Table 2
Effect concentrations in mg.L^{-1} (±standard error) of Fluoxetine (FLX) and mixtures of Fluoxetine + Activated Carbon (FLX@AC) used in the zebrafish embryo toxicity tests. It was not possible to determine an EC_{50} value for organisms exposed to FLX@100 and 1000 mg.L^{-1} of AC.

Treatment	EC_{50} (96 h)	Model (R^2)
FLX	4.22 ± 0.39	Logistic - two parameters (0.98)
FLX@AC 10 mg.L^{-1}	7.89 ± 0.67	Logistic - two parameters (0.99)

FLX@HMNC does not modify organism's behavior. The results described point out a higher interaction between FLX and HMNC; that is, HMNC is a better FLX adsorbent as compared to AC. This HMNC adsorptive property allows good zebrafish embryo development in medium containing lower FLX concentration because a greater amount of the drug is adsorbed onto the HMNC surface.

Zebrafish embryos are a good and efficient biosensor to prove the effects of FLX adsorption onto AC or HMNC. Despite its lower SSA, HMNC at low concentrations (10 mg.L^{-1}) adsorbs FLX more efficiently than AC. This is because AC and HMNC establish different electronic interactions with FLX. In the HMNC composite, the presence of CNTs covering the AC surface creates new properties or improves existing properties. The CNTs contain defects and functional groups originating from the synthesis process (Rosolen et al., 2006; Matsubara, 2010), which favor interaction with the FLX amine group and make the FLX molecules less bioavailable to the zebrafish embryos. At high concentrations (100 mg.L^{-1}), HMNC can adsorb more FLX molecules than AC, which explains the absence of behavioral changes in zebrafish embryos in the presence of FLX@HMNC. This result reinforces the data obtained for HMNC in water: despite bearing CNTs, the highest HMNC concentration in the absence of FLX is not toxic to the zebrafish. This is because CNTs are in the form of a micro/nanostructured hybrid and not in the form of free particulates. Fig. 6 represents how HMNC may have acted.

To confirm this assertion, we measured the amount of free FLX in the medium after contact with the carbonaceous adsorbent by the HPLC technique. We analyzed the different FLX, AC, HMNC, FLX@AC, and FLX@HMNC concentrations of used in embryo toxicity tests were analyzed to find the saturation point of the two carbonaceous adsorbent materials. We constructed a calibration curve on the basis of HPLC peak areas for several FLX concentrations, to obtain the standard curve with the following equation

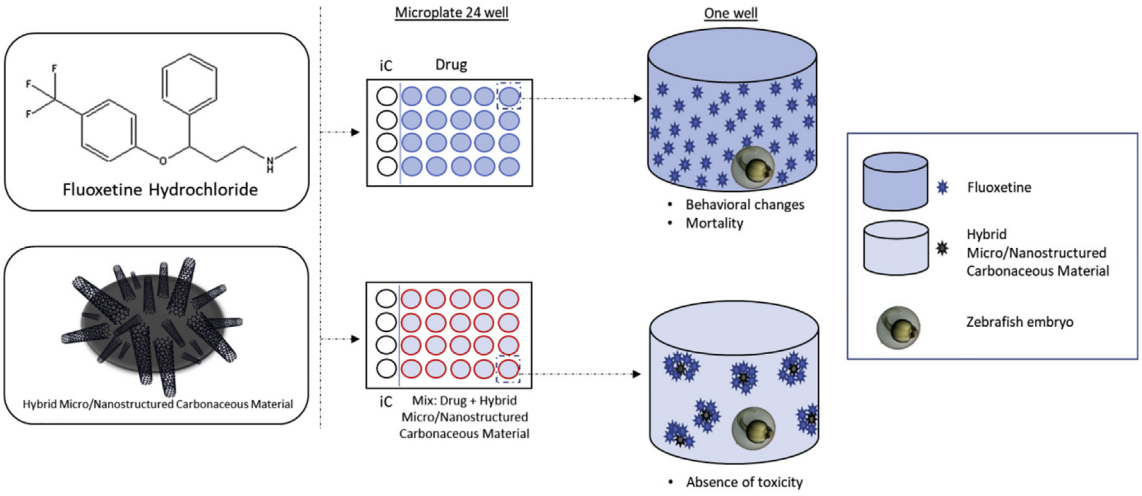

Fig. 6. Scheme of the interactions between Fluoxetine hydrochloride and Hybrid Micro/Nanostructured Carbonaceous Material. iC: internal control.

$Y = 454725^{*}X + 7501$ and R^2 of 0.9998. We used this standard curve in subsequent studies.

Fig. 7 depicts the rate of FLX adsorption onto HMNC and AC during the tests. Fig. 7 - gray color, shows that AC 1000 mg.L^{-1} only removes lower FLX concentrations (between 1 and 10 mg.L^{-1}) from the medium. When the FLX concentration increases, AC becomes saturated. Consequently, some FLX molecules remain free in the medium and are detected by the equipment. Fig. 7 – black color, demonstrates that HMNC can adsorb higher FLX quantities. Saturation starts only at higher FLX concentrations (>40 mg.L^{-1}), when HPLC detects approximately 2% of the FLX in the medium. Lastly, FTIR spectra were performed on the precipitates of FLX@AC and FLX@HMNC samples washed with methanol. The spectra obtained are typical of the carbonaceous nanostructures as prepared, and show no evidence of FLX onto micro/nanostructured surface, as can be seen in Fig. S2 (Supplementary material). Thus, confirming that FLX was successfully desorbed and the regeneration of the adsorbent is feasible.

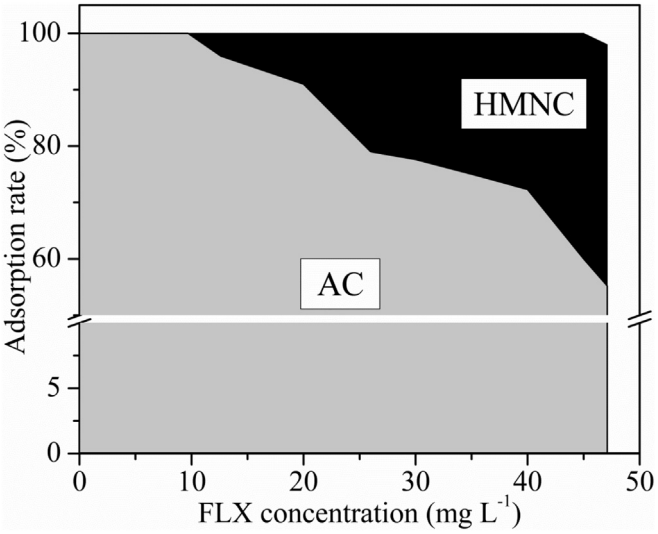

Fig. 7. (gray) Adsorption rate of various fluoxetine concentrations onto Activated Carbon 1000 mg.L^{-1}; (black) Adsorption rate of various fluoxetine concentrations onto Hybrid Micro/Nanostructured Carbonaceous Material 1000 mg.L^{-1}.

4. Conclusion

Here, we show a decrease in FLX sublethal and lethal effects on zebrafish embryos in the presence of carbonaceous adsorbent materials (AC and HMNC). The results indicate that zebrafish embryos are a good biosensor to analyze how HMNC and AC affect FLX adsorption.

HMNC and AC present low toxicity to zebrafish embryos, with LC_{50} above 1000 mg.L^{-1}, but the use of HMNC for FLX adsorption is more advantageous. The presence of CNTs on the AC surface in HMNC elicits electronic interactions that modify the properties of both AC and CNTs, giving rise to a hybrid material. The use of a hybrid composite material (micro/nanostructured carbonaceous material) enables the application of CNTs in environmental remediation without the toxicological effects attributed to carbon nanomaterials. Moreover, this approach allows an easy CNTs recovery.

HMNC proved to be a better FLX adsorbent than AC: HMNC provides the same outcome as AC at tenfold lower concentration (10 mg.L^{-1} versus 100 mg.L^{-1}, respectively) without behavioral changes.

In conclusion, HMNC resulted from a combination of activated carbon and hydrophilic carbon nanotubes can be potentially employed in water treatment or environmental remediation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.05.019.

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