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Research article

A combined chemical and ecotoxicological approach to assess nature-based solutions efficiency in removing pharmaceuticals using microcosms

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ABSTRACT

Pharmaceutical products (PP) are considered contaminants of emerging concern, and their discharge into the environment has increased due to both, human and veterinary use. These drugs entered freshwater ecosystems and remain physiologically active, posing potential negative effects on aquatic ecosystems. As conventional plants for wastewater treatment are not designed to remove most of these micropollutants, the development of strategies for sustainable management is essential and nature-based solutions (NBS) will be useful in removing pollution, simultaneously benefiting people and nature. This study aims to assess the effectiveness of PP removal of different NBSs implemented at a microcosm scale from chemical and ecotoxicological points of view. Proposed NBSs were based on floating wetlands with improvements based on the use of biochar and bioaugmentation (biofilm coating). The proposed NBSs were able to reduce up to 90 % of the total PP concentration after the whole experimental period (42 days). However, considering a regular hydraulic retention time (7 days), the higher PP removal rate (55 %), took place in the microcosms with floating wetlands enhanced with biochar (FWB), followed by the floating wetlands enhanced with biofilm-coated biochar (FWBB) (51 %) and the floating wetland (FW) (42 %). The selected NBS (FWB) has also been assessed through the toxicological approach, and the acute and behavioural effects of the wastewater were also reduced after 7 days. The proposed solution will benefit both nature and society, reducing PP pollution and enhancing water resource quality. The results obtained with the ecotoxicological approach highlight the relevance of considering the toxic cocktail, as a whole, in environmental risk assessment and the use of behavioural biomarkers for spotlighting its ecological consequences.

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1. Introduction

In 2009, Rockström et al. presented the conceptual framework of planetary boundaries that defines a series of thresholds to achieve the stability of the Earth [1]. One of these boundaries is the contamination by novel entities or contaminants of emerging concern (CECs). The term was defined by Ref. [2], which encompasses chemicals, such as drugs, and other new types of engineered materials or organisms not previously known to the Earth system, as well as natural elements, for example, heavy metals, mobilized by anthropogenic activities and accumulated at high concentrations. These toxic substances reach freshwater and marine ecosystems, likely affecting aquatic organisms, potentially causing alterations in trophic webs, and affecting ecosystems functioning and stability [3]. In addition, likely this pollution will intensify in the future, due to the increase in the activity of the chemical industry over time [4].

PP are considered CECs and their discharge into the environment has increased due to the human population increase and aging and their veterinary use [5]. These drugs that have entered freshwater ecosystems remain physiologically active, disturbing the ecosystems [6]. The number of studies on the toxic effects of PP that reach aquatic environments has increased recently [7–9] highlighting the significant ecological and health risks and the necessary research on effective mitigation strategies [10]. Therefore, PPs have contributed significantly to aquatic pollution, with urban wastewater being one of the largest pollution sources [11].

Conventional plants for wastewater treatment were not designed to remove most of the CECs [11]. Additionally, until now, they have not been subjected to specific regulations regarding their limit of presence in water, so there is no monitoring of these compounds in the different environmental compartments (water, sediments, biota) [12]. However, the European Council recently adopts new rules for more efficient treatments, and by 2045 member states will have to apply additional treatments to remove micropollutants (such as pharmaceuticals and cosmetics), known as quaternary treatment [13]. In this concern, developing strategies for sustainable water resources management is essential. Given this, some studies have called for further monitoring studies to better understand the impact on environmental contamination by pharmaceuticals and to evaluate the necessity for quaternary treatments, such as the NBS [14]. NBS are actions to protect, sustainably manage, and restore natural and modified ecosystems that address societal challenges effectively and adaptively, simultaneously benefiting people and nature [15]. Two of the key aspects in NBS are the integrity of the ecosystem-society binomial, as well as the evident link with the Sustainable Development Goals (SDGs), since it is not possible to understand the progress of society without considering the preservation of the environment and the sustainability of natural resources [16].

The aquatic plant community can purify water naturally, improving its quality. They are based on the natural processes of biological filtering of water as it passes through shallow areas and permeable soils. Emerging plants like cattails (*Thypa* sp.) absorb and eliminate nutrients such as N and P. They can also decompose contaminants and toxins from sediment and water by incorporating them into their biomass. But they also provide shelter and surface to other organisms, such as microorganisms, plankton, and fish that can help with the absorption of nutrients and the decomposition of pollutants that reach the aquatic systems [17]. So, they can be considered NBS when used to remove pollution from wastewater [18,19]. NBS designed to improve water quality, such as the artificial or constructed wetlands, are tools that allow the sustainable use of water resources and the protection of aquatic systems threatened by pollutants and whose effectiveness has been verified in a wide variety of experiences and situations [20]. The use of the plants and microorganisms' natural capacity to decompose pollutants through biological processes is known as bioremediation and is part of the processes that classify NBS as amelioration solutions (amelioration-NBS type, following [21]) and are considered a low-cost and environmentally friendly option [22]. The use of artificial or constructed wetlands as NBS, specifically floating wetlands, has been previously reported as an efficient strategy to eliminate pollutants [23]. For example, a floating treatment wetland (FTW) was used as a strategy for attenuating the pollutant concentration in a crude oil wastewater pit, being successful in removing organics, hydrocarbons, total dissolved solids, and heavy metals [24].

To improve the PP removal capacity of artificial wetlands, we can incorporate adsorbent elements, such as biochar, a carbon-rich material that can be prepared from various organic waste feedstocks, such as agricultural plant residues and sewage sludge [25]. It has been demonstrated that biochar prepared from olive oil wastes eliminated emerging contaminants from water at laboratory scale [26]. The PP elimination yields vary according to the PP typology between 40 and 96 %. The properties of these materials (physical, chemical, and biological) make them optimal for wastewater decontamination treatments [27–31]. El Barkaoui and collaborators reviewed the use of Biochar in constructed wetlands in the literature and stated that the removal efficiency of pollutants in constructed wetlands is affected by several factors, such as substrate chemical and physical properties, oxygenation, and hydraulic retention time, among others [31]. The use of biochar as an innovative amendment can enhance the removal efficiencies of nitrogen, emerging organic contaminants, and metals in constructed wetlands treatment systems, simultaneously mitigating the greenhouse effect and reducing the land requirement of these nature-based solutions [28].

Another removal enhancement strategy is based on using specific microorganisms capable of reducing PP. These organisms can be found in ecosystems with a history of pollution by PP [32] and they have been used in bioaugmentation lab experiments [33]. Although it is a little-studied field, some authors have studied the interactions between biochars and microbial biofilms, analysing the potential advantages and disadvantages of this association in the elimination of some types of contaminants [34,35]. For example, a study also confirmed that FTWs inoculated with bacteria possessed hydrocarbon degradation capability [36]. The role of the presence of strong increases in polar functional groups associated with stable biofilms in pharmaceutical contaminant purification systems still presents important unknowns that are partly intended to be analysed in this work. These functional groups are also present in microbial biomass and other authors have that these chemical groups are involved in the adsorption of pharmaceutical and personal care products [37]. On the other hand, conventional biochar obtained by simple carbonization usually has low porosity, which limits its adsorption capacity, while their chemical activation is expensive [38]. For this reason, we aimed to assess if biochar enhanced with microbial biofilm could improve its adsorption capacity against pharmaceuticals.

We hypothesised that both strategies, the use of biochar and the use of biofilm-coated biochar, enhance the floating wetland capacity of removing PP, and reduce the toxic effect from wastewater, functioning as innovative NBSs.

This study aims to assess the effectiveness of PP removal of the different NBSs implemented at microcosms scale, firstly from a chemical point of view analysing the rate of PP removal; and secondly, from an ecotoxicological point of view, analysing the toxicity of the wastewater, before and after the NBS, using standard and innovative toxicological protocols. The combination of both assessments is key to informing quantitatively and qualitatively on the NBSs' efficiency.

2. Material and methods

2.1. Chemicals and reagents

A total of 107 pharmaceuticals and some of their relevant transformation products were included in the monitoring (Table S1). The selection of the analytes was based on those commonly found in wastewater effluents from the area [39] as well as some compounds included in the European Watch List published by the European Parliament in 2022. Analytical standards (purity ≥98 %) were purchased from Dr. Ehrenstorfer (Augsburg, Germany) and Merck Group (Darmstadt, Germany). A surrogate standard solution of difenoxuron in methanol (MeOH) was used as an internal standard (IS) for extraction quality control check. Acetonitrile (ACN), MeOH, and water (LC-MS grade) were acquired from Merck Group (Darmstadt, Germany). Formic acid (FA) was supplied by Honeywell Fluka (Buchs, Switzerland). Oasis HLB SPE cartridges (200 mg, 6 mL) were acquired from Waters (Milford, MA, USA).

2.2. Biochar characterization

The ground biochar used in the study is generated from pruning biomass using the hydrothermal carbonization process and it was provided by INGELIA S.L. The company provides also the general characterization of moisture (7 %), ash content (5–10 % dry weight), total carbon (55–60 % ash-free dry weight-AFDW), total nitrogen (1–1.5 % AFDW), volatiles (45–55 % on carbon) and lower heating value (LHV 22–23 MJ/kg). The biochar bulk or apparent density is 750 kg m^{-3}.

2.3. Biofilm-coated biochar process

A ubiquitous strain isolated from wastewater from the province of Jaén [40] and registered in the Spanish collection of type cultures (CECT) was selected to evaluate its ability to form biofilms that improve the adsorption capacity of PP by the pruning biochar pellets chosen for this work. *Rhodotorula mucilaginosa* 1S1 CECT 13212 had already shown a good biosorption capacity for Ag(I) [41] based on a strong presence of hydroxyl, carbonyl, amino, methyl, and methylene functional groups, which suggested that it could provide an improvement in the adsorption of the organic molecules evaluated in this research. Using Fourier Transform Infrared Spectroscopy (FT-IR) we analysed whether biochar enhanced with microbial biofilm could improve its adsorption capacity against PP. Before starting the tests, the pellets were subjected to light sterilization at 85 °C for 48 h. Starting with a 24-h pre-inoculum on YPG medium (yeast, 10 g L^{-1}; peptone, 10 g L^{-1}; glucose, 20 g L^{-1}), batch tests were started at 27 °C and 150 rpm. The biochar was placed in a sterile mesh to prevent damage from the agitation conditions. After 10 days of growth, the pellets were washed repeatedly with 0.1 M NaCl and under shaking conditions to remove any remains of the nutrient medium. Finally, samples were taken and fixed with 2.5 % glutaraldehyde, they were dehydrated with increasing concentrations of acetone and after drying at 35 °C, they were metalized with carbon for analysis by scanning electron microscopy (SEM) with the idea of confirming the colonization of the microorganism on the surface of the biochar. In parallel, an analysis of FT-IR by attenuated total refraction (ATR) was carried out on the biochar pellets previously ground in porcelain mortar (before and after growing biofilm), to check the presence of functional groups. Both, biochar and biofilm-coated biochar, were used in batch tests to determine the adsorption of carbamazepine, diclofenac, and sulfamethoxazole under laboratory conditions (Table S2) and analysed by FT-IR. Both materials were later used in the microcosm-scale experiment framed in the NBS proposed in this work.

2.4. Microcosms setup and stabilization phase

Twenty polypropylene containers (556 x 356 × 304 mm) were used as microcosms. They were placed in the experimental wetland, an area at the University of Jaén (Spain) devoted to research under outdoor conditions. They were filled with previously dechlorinated tap water, reaching a final volume of 30 L. Floating wetlands were assembled following the supplier instructions with 6 plants of *Thypa* sp. species inserted in the plastic tile in each microcosm (QUARQ ENTERPRISE S.A.) (Microcosms' pictures in Supplementary Material Fig. S1). The microcosms were stabilized for 5 weeks, allowing the plants to develop before wastewater exposure. Microcosms were monitored weekly, using the YSI-556MPS multiparametric probe to measure: temperature (°C), pH, conductivity (mS/cm), and dissolved oxygen (%). Aquaflor fluorometer (Turner Designs) was used to measure chlorophyll-*a* content (Chl-*a*).

2.5. Microcosms wastewater exposure phase

After 5 weeks of stabilization, the microcosms were exposed to wastewater (WW) from the Santa Catalina Wastewater Treatment Plant (secondary treatment, Jaén, Spain). Static systems were used in this study. The treatments studied were as follows. **Null treatment**: microcosms without floating wetland, with WW; **Control FW**: microcosms with floating wetland with stabilization phase

water; **FW**: microcosms with floating wetland with WW; **FWB**: microcosms with floating wetland with WW, improved with biochar; **FWBB**: microcosms with floating wetland with WW, improved with biofilm coated biochar. Four replicates for each treatment were established.

In those treatments with biochar, the microcosms received 60 g of biochar distributed in 6 permeable bags located close to the *Thypha* sp. radicular section (Fig. S1). The exposure phase lasted 7 weeks, and samples were taken every 3 days during the first week, and then once per week until the end of the study: days 0, 3, 7, 14, 21, 28, 35, and 42. The chemical, physical, and biological parameters mentioned in the stabilization phase were also measured on each sampling day during the exposure phase. Further, water samples were taken from each microcosm for subsequent semiquantitative nutrient analysis (nitrate, using the VISOCOLOR™ ECO Rapid Kits; Phosphate content using QUANTOFIX™ Quick Strips. Additionally, samples were taken in 1 L amber glass bottles from each microcosm to carry out the analysis of pharmaceuticals by liquid chromatography/mass spectrometry (LC-MS). The bottles were filled with a syringe to a volume of 750 mL and closed with Teflon caps. After that, the bottles were superficially disinfected with ethanol, labelled, and finally frozen at −20 °C until analysed.

2.6. Monitoring of pharmaceuticals

2.6.1. Sample preparation

Samples were defreezed and filtered (cellulose, 0.45 μm pore size). A solid-phase extraction (SPE) procedure, based on our previous studies [39,42] was applied. Briefly, 200 mL of treated water were spiked with an IS solution and extracted using an Oasis HLB cartridge. The SPE cartridge was preconditioned with 4 mL of MeOH and 8 mL of H_2O (LC-MS grade). After sample loading, the cartridge was dried under vacuum for 30 min. The elution of the analytes was performed with two aliquots of 4 mL of MeOH. The extract was then evaporated in a water bath (37 °C) under a gentle nitrogen stream (15 psi) until dryness (30 min approx.), and reconstituted with 2 mL of MeOH. Before injection in the LC-MS, a 1:50 dilution of the extract using Milli-Q water was carried out. Therefore, the complete procedure involves a global 2:1 preconcentration of the WW sample [42].

2.6.2. Sample analysis by LC-MS and quantification

Instrumental analysis was carried out using the same conditions as previously reported [42]. A Thermo Vanquish Flex LC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Q-Exactive Orbitrap (Thermo Fisher Scientific) mass spectrometer was employed for sample analysis. A Zorbax Rapid Resolution High Definition (RRHD) Eclipse Plus C18 (2.1 mm × 50 mm, 1.8 μm, Agilent Technologies, Wilmington, DE, USA) analytical column was used for chromatographic separation. A mixture of ultrapure water (0.1 % FA, eluent A) and MeOH (0.1 % FA, eluent B) was used as the mobile phase. The gradient employed was as follows: initial conditions, 10 % B for 0.7 min; within 4.0 min, linear increase from 10 % to 50 %; within 17 min, linear increase to 95 % B; kept constant at 95 % B for 8 min; within 0.1 min decreased to 10 % B; and kept constant for column equilibration during 5 min. The total analysis run time was 30 min.

The mass spectrometer was equipped with a heated electrospray ionization source (HESI) operating in both positive and negative modes (individual analysis). The HESI parameters were as follows: sheath gas flow rate 55 units; aux gas flow rate 15 units; spray voltage 3.5 kV (2.5 kV, negative ionization); capillary temperature 300 °C; S-lens RF level 60; and aux gas heater temperature 350 °C. The detection of the compounds was performed using a combination of full-scan (FS) and data-dependent acquisition (DDA) for MS/MS spectra. For FS analysis, data were acquired at a resolving power of 70,000 FWHM; the automatic gain control (AGC) target was set at 3×10^6 with a maximum injection time (IT) of 100 ms, and a scan range from m/z 65 to 950. For DDA, resolution was 17,500 FWHM; AGC target 2×10^5; Maximum IT 100 ms; loop count 5; maximum number of ions to trigger after FS based on ion abundance (TopN) 5; isolation window 1 Da; and normalized collision energy (NCE) set at 35. Xcalibur 3.0 and Trace Finder 3.3 (Thermo Fisher Scientific) software were used for qualitative and quantitative data analysis.

The methodology was validated by assessing linearity, method quantification limits (MQLs), matrix effects, trueness (expressed as recoveries), and precision (expressed as relative standard deviations, RSDs). Detailed validation results for the 107 compounds studied can be found elsewhere [42]. For quantification purposes, the concentration of the PP was calculated by interpolating the obtained peak area in matrix-matched calibration curves (range 0.005–5 μg L^{-1}).

2.7. Toxicological assessment

Two toxicological tests were carried out using *Daphnia magna* as the test organism to evaluate the environmental risk that wastewater PP cocktails may pose to aquatic biota.

Firstly, an acute toxicity test based on the OECD protocol [43] was carried out to find the wastewater LD50 (lethal dilution) for the *D. magna* population cultured at the University of Jaén. *D. magna* was reared in mineral water in the laboratory for many years using the microalga *Scenedesmus obliquus* (Turpin) Kützing 1833 as food (Chemical Engineering Laboratory, University of Jaén). These algae were routinely maintained in 3 N-BBM*V culture media at pH 8.3–8.5 (modified from CCAP, Scotland). Both, *D. magna* and *S. obliquus* were cultured in a temperature-controlled chamber (ARALAB-600PHL-LED) and under the same conditions (20 °C and a 12:12 h light: dark cycle). Enriched mineral water (EMW) with thiamine (0.075 g L^{-1}), vitamin B12 (0.010 g L^{-1}), biotin (0.0075 g L^{-1}), and sodium selenite (0.010 g L^{-1}) was used following Díaz-Báez et al. (2008) culture protocol. WW from the Santa Catalina wastewater treatment plant effluent (WWTP) was used to prepare the treatments for the acute toxicity test: Control treatment (100 % EMW used for daphnids culture, also used to make the dilutions); 25 % WW treatment (25 % WW + 75 % EMW); 50 % WW treatment (50 % WW + 50 % EMW); 75 % WW treatment (75 % WW + 25 % EMW), and 100 % WW treatment (100 % WW). Ten daphnids (<24 h) were added to each

replicate (3 replicates in each treatment). Therefore, 150 individuals were used and maintained under the same culture conditions of light and temperature (ARALAB-600PHL-LED chamber) but without food. Survival was checked after 24 h and 48 h.

Secondly, two avoidance behaviour tests were conducted to assess the effects on *D. magna* behaviour. In the first one, we use the WW, and in the second one, we use the water from FWB microcosms taken by the end of the exposure period (day 42). We decided to use the FWB treatment as representative of the innovative NBSs proposed, and due to the limitation of only being able to perform an HeMHAS experiment. We have followed the methodology used by Moreira & collaborators [44] and the HeMHAS system (Heterogeneous Multi-Habitat Assay System). This system allows a non-forced exposure to toxicants and the analysis of the avoidance or escape behaviour, when the individuals select the most convenient/safe compartment. In the first test, the dilutions set was: i) 100 % *D. magna* culture water (EMW), ii) 50 % wastewater from the treatment plant (WW) + 50 % EMW, and iii) 100 % WW (Fig. 1a). In the second test, carried out to confirm the effect of the water from the FWB, the dilutions set was: i) 100 % EMW, ii) 50 % water from microcosms FWB +50 % EMW and iii) 100 % water from microcosms FWB (Fig. 1b). Ten adult daphnids were placed in each compartment, with the gates open allowing the movement of individuals along the dilution gradient. Individuals in each compartment were counted every half hour until reaching 4 h. The last check was carried out after 24 h. HeMHAS system allows to perform 3 replicates per trial, using 150 individuals on the whole.

2.8. Statistical analysis

The microcosm stabilization period results were analysed with a Generalized linear model (GLM) and Tukey post hoc to test differences among microcosm concerning the measured variables (physicochemical and biological variables). RStudio software was used.

A shade chart was plotted to visualize the concentration patterns of PP on the different treatments over time. A non-metric multidimensional scaling (nMDS) ordination plot, based on the Bray-Curtis similarities among treatments, was carried out; the closer points are to each other the more similar the PP composition of the different NBSs [45]. Success of the ordination was measured by a stress coefficient [46].

To detect differences in PP composition between treatments over time, a multivariate analysis of the PP data was performed using a

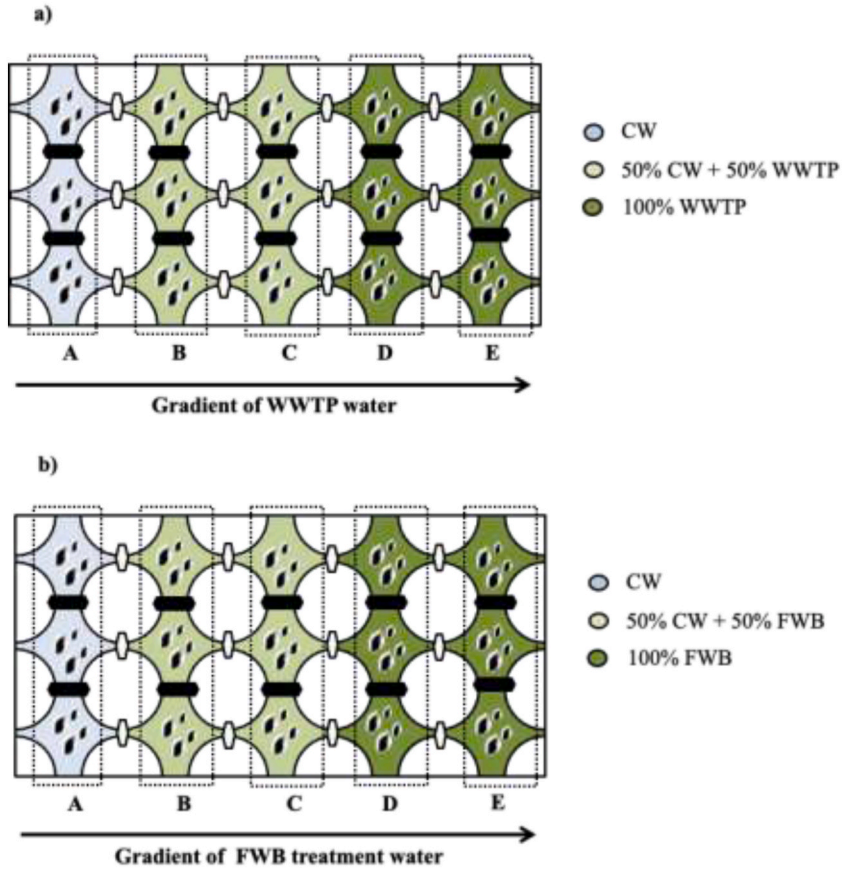

Fig. 1. Avoidance behavior experiments schemes in HeMHAS system. a) Dilution gradient using 100 % *D. magna* culture water (CW); 50 % wastewater treatment plant (WWTP) + 50 % CW and 100 % WWTP water. b) Dilution gradient using 100 % CW, 50 % water from the FWB microcosm (FWB) + 50 % CW and 100 % FWB.

Permutational Analysis of Variance (PERMANOVA). A two-way design was implemented, that is, the factor NBSs with 4 levels (Null; FW; FWB; FWBB), the factor time with 3 levels (0, 3 and 7 days) and the interaction between them. This analysis was undertaken with 9999 permutations on Bray-Curtis similarity matrices.

In addition, a Similarity Percentage Analyses (SIMPER) was performed to identify the discriminant PPs between treatments (i.e., which PP contributes to the differences between PP assemblages) [45]. PRIMER v7 software was used to perform all these analyses. Data were previously fourth root transformed in order to reduce the influence of more abundant PP and increase the influence of rarer PP in the analysis [46].

Several two-way Generalized Linear Model (GLM), with Poisson distribution and log-link function, were performed [47] to detect the potential effects of NBSs and time and their interaction on the abundances of PP.

Acute toxicity test results were analysed using the non-parametric test Kruskal-Wallis test to evaluate differences among treatments (non-normal distributed data). To perform multiple comparisons between groups, the Dunn test with Holm adjustment was applied.

Avoidance behaviour test results were analysed using analysis of variance (ANOVA) to assess differences in means among treatments (normal distributed data). To further investigate which specific groups were different, a Tukey Honest Significant Difference

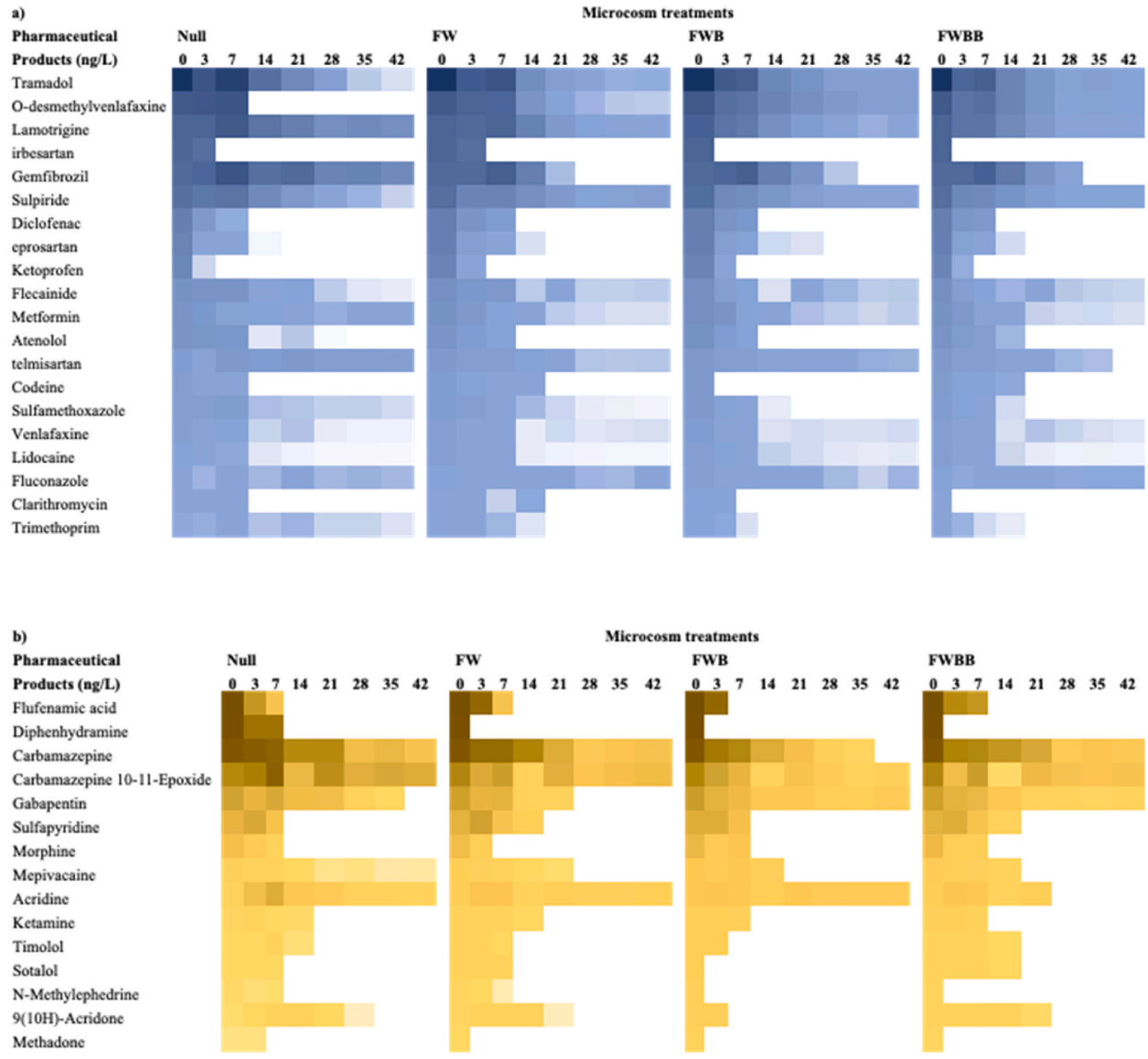

Fig. 2. Heatmaps show pharmaceutical products (PP) concentration along the exposure period (42 days) in the different microcosm treatments. The rectangular color-coded arrays indicating the intensity (in our case, concentration) of the PP. a) PP with initial concentrations in the range of 2000-100 ng L^{-1} (in blue) and b) PP with initial concentrations below 100 ng L^{-1} (in yellow). **Note:** Microcosms treatments: **Null**: microcosms without floating wetland, with wastewater; **FW**: microcosms with floating wetland with wastewater; **FWB**: microcosms with floating wetland with wastewater, improved with biochar; **FWBB**: microcosms with floating wetland with wastewater, improved with biofilm coated biochar.

(HSD) test was conducted.

3. Results

3.1. Biofilm-coated biochar process

The preliminary results obtained in the biological activation tests of the biochar demonstrated that the yeast *R. mucilaginosa* 1S1 CECT 13212 had a good capacity to promote biofilm formation on the mentioned organic support (Fig. S2), showing a strong release of exopolysaccharides (EPS) by the yeast and a uniform distribution.

On the other hand, the FT-IR spectra obtained before and after the formation of microbial biofilm showed a slight increase in the intensity of the functional groups concerning the original biochar with a predominance of hydroxyl groups associated with two regions with intervals located at 3000-3300 cm^{-1} and at 900-1200 cm^{-1}; methyl (CH_3) and methylene (CH_2) groups derived from C-H bonds in the regions between 3000 and 2800 cm^{-1} and 1300-1500 cm^{-1}; carbonyl (C=O) and amino ($^{-}$NH) groups associated with amides I and II in the region between 1500 and 1800 cm^{-1}; and phosphate groups (P-O) represented in the bands in the region located between 900 and 1300 cm^{-1}.

Table S2 shows the results obtained in the batch adsorption tests and it was observed that the biochar associated with microbial biofilm had improved the adsorption in the case of diclofenac while the other two compounds have been absorbed slightly worse. In general, the batch adsorption stage caused significant changes in the FTIR spectra (Fig. S3), including changes in intensity and shifts in the bands. The bands located between 1300 and 600 cm^{-1} underwent significant changes, and this shows that the groups associated with this region were involved in the adsorption of the pharmaceutical compounds studied.

These results allow us to confirm that the biochar alone presented a wide range of functional groups that can interact with the PP. Likewise, *R. mucilaginosa* 1S1 CECT 13212 and the microbial biofilm created have also been shown to increase the number of functional groups, leading to a potential increase in PP biosorption capacity by the biochar.

3.2. Microcosms stabilization phase and wastewater exposure phase

During the stabilization phase, the microcosm showed similar values in the physic-chemical and biological variables, allowing the macrophyte stabilization and growth (mean ± SD, Temperature: 23.98 ± 0.85 °C; D.O.: 69.39 ± 2.55 %; pH: 5.83 ± 0.25; Conductivity: 0.92 ± 0.07 mS/cm; and Chlorophyll *a*: 48.56 ± 33.58 mg L^{-1}). Concerning the exposure phase, at the end of this period (day 42), there were differences between the Null treatment and the rest of the treatments just in pH (Table S3).

3.3. Chemical assessment

The wastewater (WW) samples were from the Jaén treatment plant (115,000 inhabitants). Nine compounds showed saturated signals in the LC-MS analysis (caffeine, antipyrine, paracetamol, theobromine, theophylline, naproxen, valsartan, nicotine, and cotinine). Therefore, they were discarded from the study due to the impossibility of reinjecting diluted sample extracts for their proper quantification. Overall, a total of 39 active substances were quantified in WW samples: (2-ethylidene-1-5-dimethyl-3-3-diphenil-pyrrolidine perchlorate, 9(10h)-acridone, acridine, atenolol, bupropion, carbamazepine, carbamazepine 10-11-epoxide, citalopram, clarithromycin, cocaine, codeine, diclofenac, diphenhydramine, eprosartan, flecainide, fluconazole, flufenamic acid, gabapentin, gemfibrozil, irbesartan, ketamine, ketoprofen, lamotrigine, lidocaine, mepivacaine, metformin, methadone, morphine, N-methyl-ephedrine, O-desmethylvenlafaxine, sotalol, sulfamethoxazole, sulfapyridine, sulpiride, telmisartan, timolol, tramadol, trimethoprim, and venlafaxine). Ten of these positive findings corresponded to substances included in the Watch List for surface water monitoring

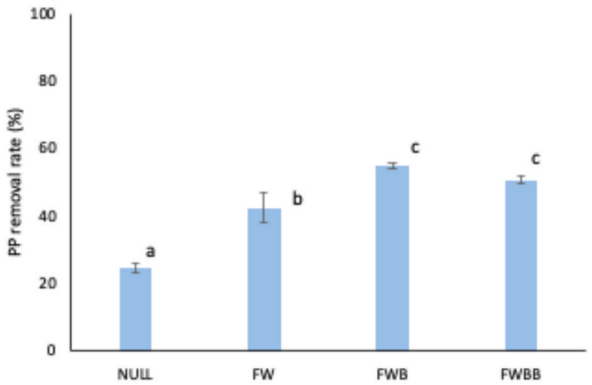

Fig. 3. Total pharmaceutical products (PP) removal rate (%) in wastewater after 7 days in the microcosms treatments: NULL: microcosms without floating wetland; FW: microcosms with floating wetland; FWB: microcosms with floating wetland, improved with biochar; FWBB: microcosms with floating wetland, improved with biofilm coated biochar. Different letters denote significant differences.

[48]: clarithromycin, diclofenac, clotrimazole, fluconazole, O-desmethylvenlafaxine, venlafaxine, metformin, ciprofloxacin, sulfamethoxazole, and trimethoprim.

After the exposure period in those microcosms without any NBS (treatment Null), 4 pharmaceutical products were not detected after 3 days (2-ethylidene-1-5-dimethyl-3-3-diphenil-pyrrolidine, bupropion, citalopram and cocaine). These PP were not included in the heat maps created for the whole experimental period (Fig. 2), where the colour intensity was used to represent PP average concentration in each treatment. The data were separated into two different concentration ranges, creating two heat maps for a better visualization of the PP removal process (below and over 100 ng L^{-1}, Fig. 2a and b respectively).

We decided to further analyse the changes in PP composition in the first 7 days of the experiment as, under WWTP regular conditions, water flows in through the artificial wetland with an average hydraulic retention time of around this lapse of time. Considering the global PP concentration, the removal rate by day 7 indicates that those NBSs with biochar showed higher efficiency. The higher PP removal rate took place in the microcosms with FWB, followed by the FWBB, and significantly different from the FW and Null treatment removal rates ($F_{(3,7)} = 72.632$; $p < 0.001$; Fig. 3). The non-metric multi-dimensional scaling (nMDS) ordination plot based on the Bray-Curtis similarities (Fig. 4) shown a gradient in PP structure from day 0 to day 7 (along MDS axis 1) and related to NBSs (along MDS axis 2). The 2-d stress level was low, 0.06, so this nMDS provides an excellent representation in reduced dimensions. The PP composition of the FWB on day 7 was different from the rest of the treatments on the same day and very different from the PP composition at the beginning of the experiment. On day 3, the composition of PP in FWB and FWBB were more similar to each other than to the rest of the treatments.

Indeed, these results agreed with a two-way PERMANOVA which detected significant differences in the PP composition between NBSs (Pseudo-$F_{(3,17)} = 25{,}88$; $p = 0.001$) and a significant effect of the exposure time (Pseudo-$F_{(2,17)} = 153.06$; $p = 0.001$). The interaction was also significant (Pseudo-$F_{(6,17)} = 6.784$; $p = 0.001$); in fact, as nMDS shown, in all NBSs, PP composition changed over time, but these differences were less noticeable for FWBB and more remarkable for FWB (Fig. 4).

Considering the individualized PP GLM results, the PP's concentrations were significantly reduced by the treatments ($p < 0.05$), just Ketamine and Sulfapyridine concentrations were not different among treatments ($\chi^2_{(3,\ 20)} = 2.43$; $p = 0.4881$ and $\chi^2_{(3,\ 20)} = 4.6101$; $p = 0.2027$, respectively).

In Table 1 the dissimilarity percentages values were presented, confirming that the higher PP removal took place in the FWB microcosms, followed by the FWBB. To elucidate which PPs contribute to the differentiation between treatments, a SIMPER was performed. Table 2 shows the discriminant PP among the wastewater treatment plant (WWTP, day 0) and the treatments at day 7. To identify the PP that contributes the most to the differentiation between groups, the focus should be on the Average Dissimilarity column; they will also tend to be the PPs with the larger abundances. In addition, to detect the best indicator of the differences between those groups, Diss/SD ratio should also be considered, since it can sometimes indicate PP that are completely absent in one group and with very consistent presence in the other, but with low abundance [45]. Checking both indicators (i.e., Av. Diss. and Diss/SD), we observed that both treatments, FWB and FWBB, were very effective in reducing or removing irbesartan, ketoprofen, clarithromycin, diphenhydramine, and metformin, among others (Table 2c–d). However, the SIMPER shows the discriminant PP among treatments in day 7 (Table 3), FWB removed more efficiently codeine, flufenamic acid, sotalol, timolol and 9(10H)-acridone, among others, than FWBB (Table 3f). All the NBS (FW, FWB, FWBB) were effective in eliminating diphenhydramine and methadone in less than 3 days (Fig. 2b; Table 3), while both PP were still in the Null microcosms by that time. Both FWB and FWBB were effective in removing completely in less than 3 days ibersartan and N-methilephedirne. FWBB was effective in removing completely in less than 3 days clarithromycin (Fig. 2a).

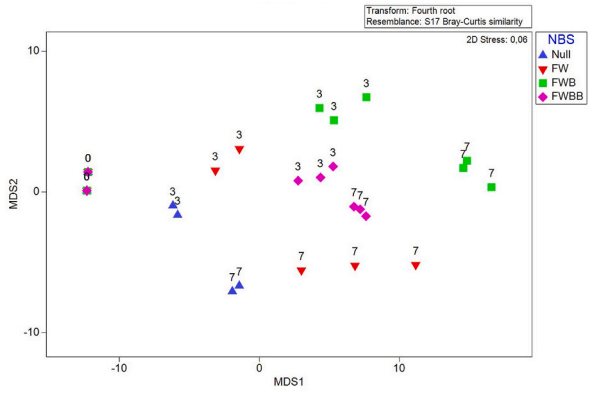

Fig. 4. Non-metric multi-dimensional scaling (nMDS) ordination plot based on the Bray-Curtis similarities. Blue triangles Null (microcosms without floating wetland), red triangle FW (microcosms with floating wetland), green square FWB (microcosms with floating wetland with biochar) and pink diamond FWBB (microcosms with floating wetland with biofilm coated microorganism). Numbers above triangles, squares and diamonds represent the sampling days (day 0, 3 and 7).

Table 1
Average dissimilarity percentage between treatments and exposure days estimated with a Similarity Percentage Analyses (SIMPER) based on the Bray-Curtis similarities.

	WW Day 0	Null-D3	Null-D7	FW-D3	FW-D7	FWB-D3	FWB-D7	FWBB-D3
WW Day 0								
Null-D3	9.6							
Null-D7	16.19	10.58						
FW-D3	12.93	6.71	15.44					
FW-D7	23.32	15.62	11.93	13.09				
FWB-D3	22.22	16.06	16.68	11.99	11.88			
FWB-D7	31.46	23.92	21.35	20.12	11.94	10.83		
FWBB-D3	21.91	14.89	15.88	10.73	10.34	8.41	13	
FWBB-D7	25.37	17.65	15.24	13.72	7.87	9.12	8.02	7.1

Note: Microcosms treatments: **WW** waste water; **Null**: microcosms without floating wetland, with wastewater; **FW**: microcosms with floating wetland with wastewater; **FWB**: microcosms with floating wetland with wastewater, improved with biochar; **FWBB**: microcosms with floating wetland with wastewater, improved with biofilm coated biochar. D3, sampling day 3. D7, sampling day 7.

3.4. Toxicological assessment

3.4.1. Acute test

After 48 h *D. magna* exposed to wastewater showed lower survival than the control (culture water). There was a 20 % reduction in *D. magna* survival in those vessels with 100 % WW (without dilution) compared to control vessels (culture water) (Fig. 5) ($\chi^2_{(4,\ 15)} = 10.5$; $p = 0.0328$).

3.4.2. Avoidance behaviour tests

In the first avoidance behaviour test and after 24 h of exposure, the number of individuals in the compartments decreases as the dilution of WW decrease too, indicating that individuals of *D. magna* are selecting compartments without WW, avoiding the exposure to it (Fig. 6a; $F_{(4,10)} = 3.781$; $p = 0.0401$).

In the second avoidance behaviour test and after 24 h of exposure, there were no differences in the number of individuals among compartments, indicating that *D. magna* individuals did not avoid the compartments with water from FWB microcosm, regardless the dilution (Fig. 6b; $F_{(4,10)} = 2.009$; $p = 0.169$).

4. Discussion

Our results showed that the three proposed NBSs: FW, FWB and FWBB, enhanced the PP removal from WW. However, the highest water quality improvement (higher PP removal rate) was obtained with FWB treatment. This finding could be attributed to the biochar adsorption capacity, which, as shown in Fig. S2, has numerous active functional groups.

The presence of the *R. mucilaginosa* 1S1 CECT 13212 in FWBB was also found to have increased the PP removal but without differences compared to FWB. In general, biological activation did not improve the adsorption capacity of the biochar, although, as mentioned (section 3.3; caption Fig. S3), it seems to do so for some of the compounds, such as tramadol, which usually involves the participation of O-H and C-O groups [49].

In general, the groups present in both the biochar and the biofilm involve the presence of oxygen and nitrogen atoms. These atoms are also present in the molecules of the compounds studied and this makes these molecules able to accept hydrogens while the groups present in the adsorbent complex can donate hydrogens forming dipole-dipole interactions known as hydrogen bonds [34]. Likewise, the fact proven in this work of the presence of polar functional groups that have both positive and negative partial charges indicates that the presence of electrostatic interactions may also be present in this case as other authors have previously demonstrated [50,51]. The electrostatic interaction has a strong dependence on the ionic strength of the medium but also on the pH, which in our case remained slightly above neutrality and would therefore be facilitating this mechanism by preventing the protonation of the functional groups allowing them to have a negative charge [51]. Another mechanism that cannot be ruled out is the presence of hydrophobic interactions, since non-polar groups tend to group, minimizing their contact with water molecules. Finally, some authors have indicated that the pore filling of biochar is also present among the bioremediation mechanisms of some pharmaceutical products [34].

However, the lack of general improvement in the response of biofilm-coated biochar might be explained by the heterogeneity of the plant matter used to manufacture the biochars (state of vegetative maturity and nature of the plant residue) and also by the heterogeneity of the resulting ground material [52]. The above could affect the particle diameter or porosity, all of which could affect the final specific surface area and, therefore the availability of the functional groups present in the biochar (biofilm-coated or not). At the

Table 2
SIMPER analysis results showing the discriminant pharmaceutical products (PP) between the WW day 0 and a) Null-D7, b) FW-D7, c) FWB-D7 and d) FWBB-D7. Columns 3 and 4: average concentration (fourth square transformed) in the pair compared. Column 5: mean dissimilarity of each PP. Column 6: the ratio of the average contribution (column 5) divided by the standard deviation (SD) of those contributions across the pair of treatments making up this average. Column 7: contribution of each PP to the total dissimilarity in the pair being compared. Column 8: cumulated percentages from column 7.

Pair of groups	PP	Aver. concen. 1 group	Aver. concen. 2 group	Diss	Diss/SD	Contrib %	Cum. %
a) WW *vs.* Null D7	*Irbesartan*	33.96	0.00	3.47	90.46	21.43	21.43
	Ketoprofen	24.33	0.00	2.49	26.62	15.36	36.79
	Diclofenac	26.77	9.93	1.72	22.26	10.63	47.41
	Eprosartan	25.94	10.89	1.54	12.75	9.50	56.91
	Metformin	20.00	13.66	0.65	8.53	4.01	60.92
	Flufenamic acid	9.93	4.81	0.52	4.44	3.23	64.15
	Gemfibrozil	32.68	37.79	0.52	14.77	3.23	67.38
	Cocaine	4.84	0.00	0.49	18.48	3.05	70.43
b) WW *vs.* FW-D7	*Irbesartan*	33.96	0.00	3.78	37.96	16.19	16.19
	Ketoprofen	24.33	0.00	2.71	23.07	11.60	27.80
	Eprosartan	25.94	9.22	1.86	8.78	7.98	35.78
	Diclofenac	26.77	14.87	1.32	4.97	5.67	41.45
	Diphenhydramine	9.96	0.00	1.11	31.45	4.75	46.20
	Metformin	20.00	11.41	0.95	11.67	4.09	50.29
	Atenolol	19.48	11.14	0.93	2.46	4.00	54.29
	Clarithromycin	10.80	3.47	0.82	1.36	3.53	57.83
	Flufenamic acid	9.93	2.64	0.82	1.70	3.50	61.33
	Tramadol	44.81	37.55	0.81	1.83	3.47	64.79
	Sulpiride CRS	31.46	24.81	0.74	6.96	3.18	67.97
	Venlafaxine	14.05	8.79	0.59	7.79	2.51	70.48
c) WW *vs.* FWB-D7	*Irbesartan*	33.96	0.00	4.04	96.94	12.85	12.85
	Ketoprofen	24.33	0.00	2.90	27.60	9.21	22.06
	Eprosartan	25.94	10.31	1.86	10.36	5.91	27.97
	Codeine	15.26	0.00	1.82	53.63	5.77	33.74
	Clarithromycin	10.80	0.00	1.29	34.48	4.09	37.83
	Diclofenac	26.77	16.66	1.20	7.22	3.83	41.65
	Sulpiride CRS	31.46	21.43	1.19	5.97	3.80	45.45
	Diphenhydramine	9.96	0.00	1.19	47.83	3.77	49.22
	Tramadol	44.81	34.84	1.18	2.69	3.77	52.98
	Flufenamic acid	9.93	0.00	1.18	9.37	3.76	56.74
	Flecainide	20.72	10.88	1.17	8.19	3.72	60.46
	Metformin	20.00	10.67	1.11	15.06	3.53	63.99
	Atenolol	19.48	10.91	1.02	11.80	3.24	67.23
	Sulfamethoxazole	15.13	7.37	0.92	5.10	2.94	70.17
d) WW *vs.* FWBB-D7	*Irbesartan*	33.96	0.00	3.85	101.18	15.19	15.19
	Ketoprofen	24.33	0.00	2.76	27.86	10.88	26.07
	Eprosartan	25.94	10.44	1.76	13.14	6.93	33.00
	Clarithromycin	10.80	0.00	1.23	34.52	4.83	37.83
	Diclofenac	26.77	16.28	1.19	9.34	4.69	42.52
	Tramadol	44.81	34.82	1.13	2.68	4.46	46.98
	Diphenhydramine	9.96	0.00	1.13	48.89	4.45	51.44
	Flecainide	20.72	11.15	1.08	11.71	4.28	55.71
	Metformin	20.00	10.68	1.06	21.13	4.17	59.88
	Sulpiride CRS	31.46	22.34	1.03	7.52	4.08	63.96
	Atenolol	19.48	13.13	0.72	7.54	2.84	66.81
	O-desmethylvenlafaxine	36.47	30.41	0.69	7.80	2.71	69.52
	Venlafaxine	14.05	8.57	0.62	8.27	2.45	71.97

Note: WW: wastewater from the treatment plant effluent; **Null**: microcosms without floating wetland, with wastewater; **FW**: microcosms with floating wetland with wastewater; **FWB**: microcosms with floating wetland with wastewater, improved with biochar; **FWBB**: microcosms with floating wetland with wastewater, improved with biofilm coated biochar. D3, sampling day 3. D7, sampling day 7.

same time, this heterogeneity could affect biofilm formation by *R. mucilaginosa* 1S1, so the coat may not have been uniform. Finally, all of this could mean that the predominant factor in PP adsorption was the specific characteristics of the ground biochar in each case, above the biological activation caused by the microbial biofilm. To what extent it is interesting to biologically coat a biochar and quantify its final contribution to PP adsorption should be studied more thoroughly in the future since the data suggest that a longer activation over time can generate much more active and stable biofilms in real conditions which can undoubtedly significantly increase the adsorption capacity of organic molecules by biochar.

During the stabilization period (no exposure) all microcosms showed similar environmental and biological variable values, which allows us to defend the experimental design and initial conditions for the later exposure period. While, at the end of the exposure to wastewater, the microcosms showed statistical differences just in pH, being NULL microcosms which showed higher pH values. The other treatments with NBS showed similar data. So, biochar and biofilm-coated biochar addition to floating wetlands did not generate

Table 3
Results of SIMPER analysis showing the discriminant pharmaceutical products (PP) between pair of groups with the different treatments (Null, FW, FWB, FWBB) on **day 7**. Column 1: pairs being compared. Column 2: PP. Columns 3 and 4: average concentration (fourth square transformed) in both groups of the pair being compared. Column 5: mean dissimilarity of each PP. Column 6: the ratio of the average contribution (column 5) divided by the standard deviation (SD) of those contributions across the pair of treatments making up this average. Column 7: contribution of each PP to the total dissimilarity in the pair being compared. Column 8: cumulated percentages from column 7.

Pair of groups	PP	Aver. concen. 1 group	Aver. concen. 2 group	Diss	Diss/SD	Contrib %	Cum. %
a) Null D7 vs. FW-D7	*Diphenhydramine*	8.75	0.00	1.13	29.65	9.44	9.44
	Atenolol	18.41	11.14	0.94	2.08	7.90	17.34
	Clarithromycin	10.38	3.47	0.90	1.29	7.56	24.90
	Sulfamethoxazole	16.27	10.46	0.75	4.38	6.29	31.19
	Sulpiride CRS	30.50	24.81	0.73	6.29	6.15	37.35
	Diclofenac	9.93	14.87	0.64	2.09	5.33	42.67
	Telmisartan	17.64	12.76	0.63	3.46	5.29	47.96
	Tramadol	41.79	37.55	0.55	2.60	4.60	52.56
	Flufenamic acid	4.81	2.64	0.55	4.37	4.60	57.16
	Venlafaxine	13.01	8.79	0.54	4.78	4.56	61.72
	O-desmethylvenlafaxine	37.59	33.52	0.53	2.37	4.40	66.12
	Morphine	3.26	0.00	0.42	6.28	3.51	69.63
	Flecainide	19.64	16.95	0.35	2.24	2.93	72.56
b) Null D7 vs. FWB-D7	*Codeine*	10.56	0.00	1.47	60.60	6.89	6.89
	Clarithromycin	10.38	0.00	1.44	96.92	6.77	13.65
	Sulpiride CRS	30.50	21.43	1.26	5.48	5.92	19.57
	Sulfamethoxazole	16.27	7.37	1.24	7.10	5.81	25.38
	Lamotrigine	36.68	27.90	1.22	11.53	5.73	31.11
	Flecainide	19.64	10.88	1.22	8.66	5.71	36.81
	Diphenhydramine	8.75	0.00	1.22	51.85	5.70	42.52
	O-desmethylvenlafaxine	37.59	29.40	1.14	6.60	5.34	47.85
	Atenolol	18.41	10.91	1.04	6.58	4.88	52.74
	Tramadol	41.79	34.84	0.97	7.66	4.53	57.27
	Diclofenac	9.93	16.66	0.94	4.79	4.39	61.65
	Trimethoprim	10.39	3.94	0.90	16.53	4.20	65.86
	Venlafaxine	13.01	6.74	0.87	3.69	4.09	69.95
	Telmisartan	17.64	11.78	0.82	6.08	3.82	73.77
c) Null D7 vs. FWBB-D7	*Clarithromycin*	10.38	0.00	1.37	108.90	8.96	8.96
	Diphenhydramine	8.75	0.00	1.15	54.01	7.56	16.52
	Flecainide	19.64	11.15	1.12	16.99	7.33	23.85
	Sulpiride CRS	30.50	22.34	1.07	6.97	7.05	30.90
	Lamotrigine	36.68	28.88	1.03	5.57	6.74	37.64
	O-desmethylvenlafaxine	37.59	30.41	0.94	5.23	6.20	43.84
	Tramadol	41.79	34.82	0.92	7.25	6.02	49.86
	Sulfamethoxazole	16.27	9.78	0.85	8.12	5.61	55.47
	Diclofenac	9.93	16.28	0.84	6.70	5.48	60.95
	Trimethoprim	10.39	5.05	0.70	8.21	4.62	65.57
	Atenolol	18.41	13.13	0.69	4.29	4.56	70.13
d) FW-D7 vs. FWB-D7	*Codeine*	11.02	0.00	1.72	33.10	14.44	14.44
	Lamotrigine	34.16	27.90	0.98	7.21	8.18	22.63
	Flecainide	16.95	10.88	0.94	4.82	7.90	30.53
	O-desmethylvenlafaxine	33.52	29.40	0.64	3.64	5.37	35.90
	Trimethoprim	7.76	3.94	0.60	12.16	4.99	40.88
	Sulpiride CRS	24.81	21.43	0.53	1.97	4.41	45.30
	Clarithromycin	3.47	0.00	0.53	0.67	4.41	49.71
	Sulfamethoxazole	10.46	7.37	0.48	2.16	4.02	53.73
	9(10H)-Acridone	2.95	0.00	0.46	22.17	3.86	57.59
	Atenolol	11.14	10.91	0.42	1.94	3.55	61.14
	Tramadol	37.55	34.84	0.42	2.00	3.51	64.64
	Diclofenac	14.87	16.66	0.40	1.60	3.39	68.03
	Flufenamic acid	2.64	0.00	0.40	0.67	3.36	71.40
e) FW-D7 vs. FWBB-D7	*Flecainide*	16.95	11.15	0.85	6.25	10.76	10.76
	Lamotrigine	34.16	28.88	0.77	3.62	9.83	20.59
	Flufenamic acid	2.64	7.00	0.75	1.61	9.56	30.15
	Morphine	0.00	3.44	0.51	11.19	6.42	36.57
	Clarithromycin	3.47	0.00	0.50	0.67	6.29	42.86
	O-desmethylvenlafaxine	33.52	30.41	0.45	2.39	5.77	48.63
	Atenolol	11.14	13.13	0.40	1.02	5.07	53.70
	Trimethoprim	7.76	5.05	0.40	4.60	5.05	58.75
	Tramadol	37.55	34.82	0.40	1.96	5.03	63.78
	Sulpiride CRS	24.81	22.34	0.36	1.85	4.58	68.36
	Diclofenac	14.87	16.28	0.34	1.67	4.31	72.66
f) FWB-D7 vs. FWBB-D7	*Codeine*	0.00	10.93	1.76	67.12	21.93	21.93

(*continued on next page*)

Table 3 (*continued*)

Pair of groups	PP	Aver. concen. 1 group	Aver. concen. 2 group	Diss	Diss/SD	Contrib %	Cum. %
	Flufenamic acid	0.00	7.00	1.13	5.88	14.04	35.98
	Sotalol	0.00	2.80	0.45	38.84	5.63	41.60
	Timolol	0.00	2.70	0.44	11.05	5.42	47.03
	9(10H)-Acridone	0.00	2.64	0.43	16.28	5.30	52.33
	Sulfamethoxazole	7.37	9.78	0.39	2.03	4.85	57.18
	Atenolol	10.91	13.13	0.36	2.28	4.44	61.61
	Venlafaxine	6.74	8.57	0.30	1.12	3.70	65.31
	Sulpiride CRS	21.43	22.34	0.26	1.21	3.21	68.52
	Carbamazepine 10-11-Epoxide	5.23	6.82	0.26	2.15	3.20	71.73

Note: Microcosms treatments: **Null**: microcosms without floating wetland, with wastewater; **FW**: microcosms with floating wetland with wastewater; **FWB**: microcosms with floating wetland with wastewater, improved with biochar; **FWBB**: microcosms with floating wetland with wastewater, improved with biofilm coated biochar. D7, sampling day 7.

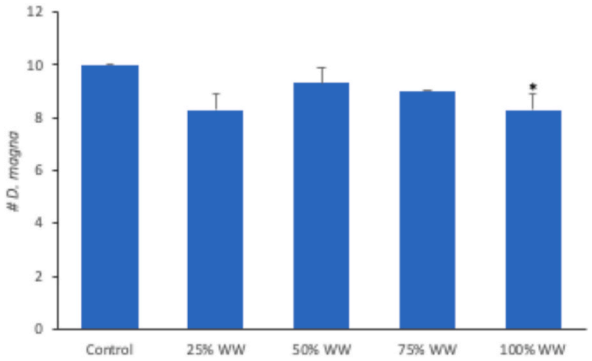

Fig. 5. Acute toxicity test results showing alive *D. magna* individuals in vessels after exposure for 48h in wastewater (WW) dilutions. Control: 100 % *D. magna* culture water. Asterisk (*) means significant differences with control vessels.

differences between NBS treatments, at least for the measured environmental variables.

Several PP were removed in the microcosms with no NBS (Null treatment). This is consistent with previous studies describing that some organic contaminants suffer photodegradation through abiotic reactions under outdoor conditions [53,54].

Diverse PP removal efficiency was observed among treatments. Although PP concentration decreased at all NBS microcosms, the FWB treatment was the most effective. The chemical assessment allows us to select the most efficient treatment from those proposed. The use of floating wetlands enhanced with biochar at a microcosms scale and the confirmation of its PP removal capacity allows us to defend it to be used at a larger scale. Several PP were completely removed in FWB by day 7 (e.g., clarithromycin, codeine, flufenamic acid, irbesartan, ketoprofen). Besides this, other PP showed high concentration reduction rates (e.g., 84 % for eprosartan and 76 % sulfamethoxazole).

From a qualitative point of view, we would like to remark that FWB reduced to zero some PP that are relevant in terms of the environmental risk assessment. Some of them due to the great demand and use, such as codeine (analgesic), flufenamic acid (analgesic), sotolol, and timolol (cardiac condition and hypertension), and others, such as clarithromycin, due to its potential role in worsening the current antimicrobial resistance crisis [55]. Similarly, the FWBB treatment showed great effectivity in the long term by removing acridine in 21 days.

It is important to highlight that although there were no differences in the total PP removal rate between FWB and FWBB, it is necessary to consider the extra inputs needed to create the biofilm (we need to consider the specialized staff's working hours, the extra facilities such as temperature-controlled chamber and the energy in terms of electricity for create and maintain the biofilm growth conditions). These disadvantages allow us to discriminate in favour of the use of the NBS based in the floating wetlands enhanced with biochar without biofilm, at least under the conditions used in this study. In addition, although out of this study's aim, a long-term treatment performance needs to be considered in future research lines to provide an integrated cost-benefit NBS assessment.

Regarding NBS and pharmaceutical pollution removal, several research gaps to be addressed have been claimed, including the ecotoxicity evaluation of NBSs' effluents [56]. The ecotoxicological approach considered in this study has several advantages confronting the chemical assessment. First of all, we have confirmed the lethal effect of the WWTP effluent as a cocktail of contaminants. We cannot give any responsibility to a specific PP but to the toxic mixture. If the WWTP effluent can affect zooplankton organisms to the extent of 20 % of the population, that means great environmental impact at that specific point. Although the natural dilution process should always be present, in areas where the streams and rivers' flow decrease largely in summer (seasonally), as in the Mediterranean region [45,57,58], special actions should be taken to reduce PP concentrations in WWTP effluents as a point-source of pollution. The NBS, as the ones we proposed, would be an option to reduce the environmental risk of WWTP effluent due to PP. The

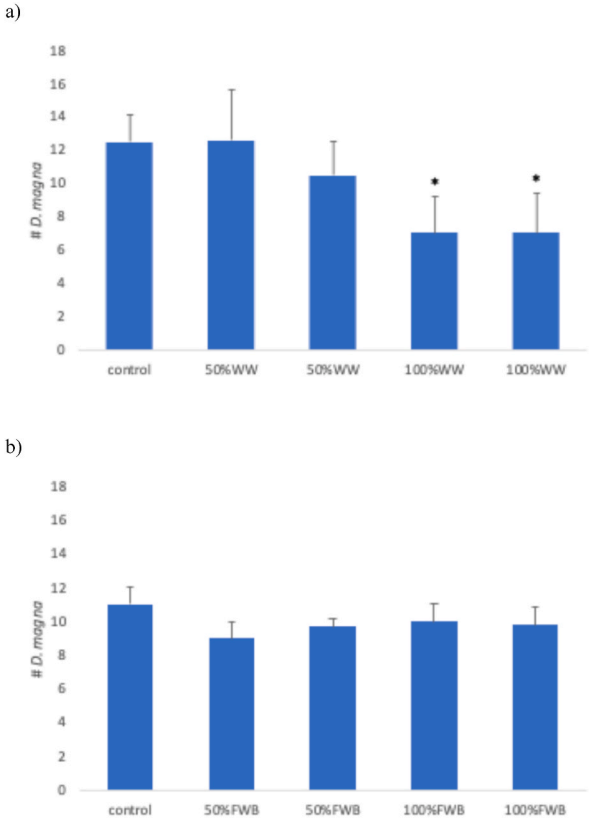

Fig. 6. Avoidance behaviour tests. Mean *D. magna* individuals in HeMHAS compartments after 24 h exposure, a) using waste water from the treatment plant (WW) and b) using water from the floating wetland with biochar microcosms (FWB). Asterisk (*) means significant differences with control compartments (Control: 100 % *D. magna* culture water).

changes and variability in rainfall predicted by the Climate Change projections will affect surface water flows and will increase pollutant concentrations, reducing water quality [59].

In addition, the toxicological assessment using a behavioural biomarker, such as avoidance behaviour, informs about the organisms' reaction to the toxic cocktail, and, therefore, gives additional and ecologically relevant information. Chemical cues are used by aquatic organisms in several ways, such as to search for food or avoid predators, but most of them to select optimal habitats [60]. WWTP effluents will create areas that the organism will try to avoid (depending on the organism's capacity to move or to escape), which can lead to specific habitat selection and, therefore, uneven organisms' spatial distribution [61,62]. Besides, these direct effects of pharmaceuticals on the traits and abundance of one species can cascade through a community, indirectly affecting other species [6]. In our case, we have confirmed that zooplankton organisms are avoiding those compartments with wastewater, while if the wastewater passes through the NBS (in our case, FWB), the mentioned behaviour does not occur. That means that the NBS reduces the negative effect of wastewater, likely PP polluted, on aquatic ecosystems. This kind of bioassays are even more relevant considering that many transformation products are not included in standard chemical analyses and, sometimes, they are more toxic than the parent product [56]. The ecotoxicological assessment shows the environmental risk reduction when NBSs are implemented and informs about their efficiency. Moreover, behaviour is considered a non-destructive biomarker that can be used as an early warning signal before major environmental impacts occur [63].

Although the present study has some limitations (such as the use of static systems microcosms and testing the effects just on one type of zooplankton organism) we provided scientific evidence on the NBS efficiency in PP pollution removal. Furthermore, the efficiency has been confirmed not only with the chemical approach but also with the toxicological approach, presenting the biological repercussions beyond the concentration data. With this ecotoxicological approach, we also highlight the relevance of considering the toxic cocktail, as a whole, in risk assessment. As cost-effective systems, they are also feasible solutions for regions with limited financial resources [64]. For more severe cases, with highly toxic pollutants, NBS can help through the implementation of hybrid systems, e.g. floating wetlands working in conjunction with subsurface systems and sand filtration systems, which will improve the final quality of the treated water [65].

5. Conclusion

The proposed nature-based solutions were efficient in reducing PP concentration from wastewater (over 40 % removal rate) and reducing its negative effects, as a toxic "cocktail" on aquatic organisms. The results presented in this study give scientific evidence on the usefulness of the proposed NBSs as quaternary treatments and their likely role in solving global water-related environmental problems concerning micropollutants. The best performance was obtained with the floating wetlands enhanced with biochar within a lapse consistent with standard hydraulic retention times (Fig. 3). Bioaugmentation with the microorganisms used in this study did not improve the global efficiency in reducing pharmaceuticals pollution, at least under our conditions. It is crucial to consider the improvement of the wastewater treatment plants' sustainability with quaternary treatments funded by NBS. Further research must be undertaken to increase NBSs' efficiency, reduce costs, and break down technical and political barriers to improve their implementation.

CRediT authorship contribution statement

María Rodríguez Santamarina: Data curation, Writing – original draft, Investigation. **Bienvenida Gilbert-López:** Data curation, Investigation, Writing – review & editing. **Ana Belén Martínez-Piernas:** Investigation, Data curation, Writing – review & editing. **Antonio Jesús Muñoz-Cobo:** Investigation, Data curation, Writing – review & editing. **María Eugenia López-Valcárcel:** Investigation, Data curation, Writing – review & editing. **Raquel Jiménez-Melero:** Formal analysis, Data curation, Writing – review & editing. **Gema Parra:** Writing – review & editing, Supervision, Methodology, Data curation, Writing – original draft, Resources, Investigation, Conceptualization, Validation, Project administration, Funding acquisition.

Data availability

Data will be made available on request and, partially at the institutional repository of scientific production of the University of Jaén (RUJA).

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e43899.

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