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Research article

Efficiency and ecotoxicity of activated biochar in the treatment of artificial wastewater contaminated by pharmaceuticals

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ABSTRACT

Pharmaceuticals are emerging contaminants of global concern due to potential ecotoxicity and persistence in wastewater. Since conventional wastewater treatment plants are not designed to remove micropollutants and the removal efficiency varies compound-specifically, pharmaceuticals pose a risk in the recipient aquatic environments. Adsorption by solid materials such as activated biochar has been suggested to offer a practical removal method. However, not much is known about the environmental risks of the adsorbents used in wastewater treatment. This study aimed to study the efficiency of activated biochar (ACB) to remove low and high concentration of specific pharmaceuticals including diclofenac (DI), tetracycline (TE), and cephalexin (CEP) from Milli-Q water (MQ) and artificial wastewater (AWW). Furthermore, the study evaluated the ecotoxicity of these pharmaceuticals, as well as pristine ACB and ACB loaded with pharmaceuticals (ACB-LP), in both MQ and AWW using *Daphnia magna*. The adsorbate concentration and matrix affected ACB's removal efficiency. Weaker adsorbent-adsorbate interactions and mass transfer resistance at lower adsorbate concentrations, along with interactions between wastewater constituents and pharmaceuticals were the leading factors contributing to this reduction. These experimental observations indicate practical considerations for using adsorbents in operational wastewater settings. Furthermore, ACB-LPs generally exhibited lower toxicity compared to ACB, attributed to the saturation of free binding sites and reduced adhesion to daphnids. This study highlights the importance of examining the environmental risks of adsorbent materials used in wastewater treatment, particularly given their anticipated future use.

1. Introduction

The presence of pharmaceuticals in the aquatic environments has raised concerns about their effect on ecosystem integrity (Yang et al., 2017; Priya et al., 2022). Detected concentrations of these compounds in drinking water, ground water, and surface water range from ng L^{-1} to μg L^{-1} (Yang et al., 2017; Priya et al., 2022). But even at low concentrations they may cause negative effects on human health and the environment (Yang et al., 2017). Non-steroidal anti-inflammatory drugs (NSAIDs), an extensively used group of pharmaceuticals, are known for inducing harmful chronic and acute effects on indigenous flora and fauna (Fent et al., 2024). Diclofenac (DI), the most frequently detected NSAID, has been linked to the decline of vultures in South Asia (Fent et al., 2024).

Similarly, antibiotics such as tetracycline (TE) and cephalexin (CEP), are highly prescribed worldwide and have broad antibacterial activity (X. Zhang et al., 2021a; K. Liu et al., 2022a). The World Health organization has categorized these resistance bacteria among the most important threats associated with human health in the 21st century (World Health Organization, 2014). The persistence of pharmaceuticals in the environment depends on their chemical properties and the characteristics of the receiving environment (Chaturvedi et al., 2021). For instance, continuous emissions of pharmaceuticals from wastewater treatment plants may result in higher concentrations in range of mg L^{-1} in river resources (Lim et al., 2017), leading to potential adverse effects in aquatic organisms (Brozinski et al., 2013). Additionally, higher concentrations of pharmaceuticals have been observed during winter, highlighting the significant impact of environmental factors, such as temperature, on their persistence and degradation rates

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(Lindholm-Lehto et al., 2016).

These pharmaceuticals are released into water bodies through various pathways, including human medical waste, animal husbandry, manufacturing sites, and agricultural and aquaculture activities (Patel et al., 2019; Chaturvedi et al., 2021). The mentioned sources contribute to the high concentrations of DI, TE, and CEP in both the environment and wastewater (Patel et al., 2019; Priya et al., 2022; Khumalo et al., 2023) with levels ranging from 1.2–12.4, 0.05–12.3, and 0.1–18.4 μg L^{-1}, respectively (Yu et al., 2016; Monteiro et al., 2018; Xu et al., 2021; Khumalo et al., 2023). Various removal methods for these therapeutic compounds, such as physical treatment, chemical remediation, thermal treatments, and biological methods, have been studied (Patel et al., 2019; Vinayagam et al., 2022). However, many of the approaches are limited due to incomplete removal, toxic sludge production and high operational costs (Patel et al., 2019). Adsorption is considered as a cost-effective and an environmentally friendly alternative (Shirani et al., 2020; Priya et al., 2022). This approach is favored through using biomass-derived adsorbents due to their availability, high efficiency, and simple preparation (Shirani et al., 2020; Juela, 2022). Biomass is converted to biochar through pyrolysis under inert atmosphere (Juela, 2022). Activated biochar which is produced using chemicals such as NaOH increase the removal efficiency of the biochar (Shirani et al., 2020).

Despite its benefits, biochar has detrimental effects on aquatic organisms possibly associated with mortality, diminished nutrient availability and cellular damage (Oleszczuk et al., 2013; Zhang et al., 2019; Woermann and Sures, 2020). The management of biochar post-adsorption is important since its release into waterbodies can harm the aquatic organisms, depending on factors such as the specific organisms involved, exposure period, environmental conditions, preparation method and adsorbent concentration (Freixa et al., 2018; Lee et al., 2022; K).

Daphnia magna is a keystone organism in most freshwater ecosystems (Altshuler et al., 2011). These filter feeders are widely used in ecotoxicological assays as bioindicator species due to their high sensitivity to environmental stressors, rapid response and easy maintenance (Wei et al., 2008; Altshuler et al., 2011). The adult *D. magna* can ingest particles up to 70 μm in size (Ebert, 2005). The particle size of biochar, and subsequently activated biochar, may vary from nanometers to millimeters (Alghamdi et al., 2020; Kroeger et al., 2021), based on different factors such as the raw material and pyrolysis conditions (temperature, heating rate, retention time and pressure) (O'Laughlin and McElligott, 2009). Consequently, small-sized particles of activated biochar used for wastewater treatment may be ingested by several aquatic species, including *D. magna*. To the best of our knowledge, no information is available regarding the ecotoxic effects of biomass-based adsorbent materials, particularly activated biochar, used in wastewater treatment on *Daphnia magna*. This was addressed by considering environmentally relevant pharmaceutical concentrations in the presence of other wastewater constituents.

Optimizing the experimental setup to achieve high removal efficiency of the adsorbent in various matrices is crucial for its potential application in wastewater treatment plants. Additionally, investigating the possible toxicity of the adsorbent after the adsorption process in wastewater treatment is important for protecting aquatic biota and the integrity of aquatic ecosystems. These studies contribute to the development of wastewater treatment practices that are safer and more sustainable for the environment.

In our earlier research, activated biochar (ACB) was obtained through pyrolysis of a forest plant in an inert atmosphere. Moreover, the efficiency of the ACB in removing high concentrations of pharmaceuticals from Milli-Q water (MQ) in batch and column studies has already shown promising results (Shirani et al., 2020).

The first aim of this study was to compare the efficiency of ACB to remove different concentrations of pharmaceuticals from MQ water and artificial wastewater (AWW). The second aim was to investigate the toxicity of the selected pharmaceuticals (DI, TE, and CEP) on *D. magna* and to compare the potential acute adverse effects of different doses of ACBs and ACB-LPs on these organisms.

2. Material and methods

2.1. Materials

2.1.1. Pharmaceuticals

Diclofenac sodium (DI; purity of 98%) was obtained from Fisher Scientific. Tetracycline hydrochloride (TE; purity ≥95%), cephalexin (CEP), hydrochloric acid (37%) and sodium hydroxide (97%) were purchased from Sigma Aldrich and were of analytical grade. The pK_a values for DI, CEP, and TE differ from each other (Table 1).

2.1.2. Artificial wastewater

The AWW was composed of $MgSO_4{\cdot}7H_2O$ (2 mg L^{-1}), $CaCl_2{\cdot}2H_2O$ (4 mg L^{-1}), $NaHCO_3$ (96 mg L^{-1}), KCl (4 mg L^{-1}), K_2HPO_4 (0.28 mg L^{-1}), NaCl (7 mg L^{-1}), urea (6 mg L^{-1}), $CaSO_4{\cdot}2H_2O$ (60 mg L^{-1}), $MgSO_4$ (60 mg L^{-1}), meat extract (22 mg L^{-1}) and peptone (32 mg L^{-1}) dissolved in MQ (Polo-López et al., 2012).

2.1.3. Activated biochar

After the collection of *Anthriscus sylvestris,* a species from the Apiaceae family known as cow parsley, they were rinsed and oven-dried at 45 °C for 24 h. Followingly, the dried plants were placed in a muffle furnace (Nabertherm GmbH, LT 9/11/B410, 30–30000 °C, Germany) for 15 min at a fixed temperature of 300 °C. The next stage was the chemical modification of the obtained material using 4 M NaOH. The material was then thermally activated in the furnace at 800 °C, washed (with MQ and 0.1 M HCl), and finally dried overnight. The final material was referred as activated biochar (ACB) (Shirani et al., 2020). Briefly outlined in this context, the specific details of the ACB preparation process are covered in our prior paper (Shirani et al., 2020). We selected *Anthriscus sylvestris* as the source material for the preparation of ACB due to its local availability, cost-effectiveness, and sustainable nature. The high lignocellulosic content of this plant leads to the formation of biochar with a high surface area, porosity, and a variety of functional groups, enhancing its adsorption capacity for pharmaceuticals.

2.1.4. Daphnia magna

D. magna ephippias (Aboatox Oy, Masku, Finland) were purchased and stored at 4 ± 2 °C until their use. To obtain D. magna neonates, the ephippias from the vials were rinsed with AFW to remove impurities, microsieved, and transferred to petri dishes containing pre-aerated AFW (hardness: $Ca^{2+} + Mg^{2+} = 2.5$ mM) and incubated for 3 day at 20 ± 1 °C under continuous light regime (OECD, 2004). The pH and the temperature of AFW were 7.2 and 20 ± 1 °C, respectively. Ephippias were monitored for hatching, which occurred after 72 h, and the neonates were fed with spirulina powder approximately 2 h before the experiment to avoid starvation.

2.2. Methods

2.2.1. Experimental setup

First, the efficiency of ACB to remove high concentrations of pharmaceuticals (10, 20 and 40 mg L^{-1}) in AWW and low concentrations of pharmaceuticals (0.001, 0.005, 0.025, 0.05, and 0.1 mg L^{-1}) in MQ was examined. These experiments were performed using 0.1 g L^{-1} of ACB in 25 mL polyethylene tubes that were agitated on the roller shaker for 24 h. After this period, the concentrations of the pharmaceuticals and the control treatments were measured using standard analytical methods (section 2.3). All experiments were conducted in duplicate for each sample and their arithmetic mean values and standard deviation were calculated. According to the following formulae (1) and (2), the removal efficiency (%) and equilibrium capacity were calculated, respectively:

Table 1
Selected pharmaceuticals characteristics.

Pharmaceutical	Acronym	CAS number	Molecular weight	Molecular formula	Water solubilty (mg mL^{-1})	Stability	$LogK_{ow}$	pK_a	Ref
Diclofenac Sodium	DI	15,307-79-6	318.13	$C_{14}H_{11}Cl_2NO_2$.Na	50	Stable, Hygroscopic	0.70	4.20	Manu (2013)
Tetracycline hydrochloride	TE	64-75-5	480.90	$C_{22}H_{24}N_2O_8$.HCl	20	–	−1.30	3.30, 7.46, 8.94	(Qiu et al., 2021; del Olmo et al., 2023)
Cephalexin	CEP	23,325-78-2	365.40	$C_{16}H_{17}N_3O_4S$. H_2O	13.5 at 25 °C. 120 at pH 2.3 at 37 °C.	Acid stable	0.97	Acidic: 5.20 Basic: 7.30	Rahim (2018)

$$Removal\ efficiency\ \% = \frac{(C_i - C_e)}{C_i} \times 100 \quad (1)$$

$$q_e\left(mg\ g^{-1}\right) = \frac{V(C_i - C_e)}{W} \quad (2)$$

In which the initial concentration of DI, TE and CEP is C_i (mg L^{-1}) and the concentration of the pharmaceuticals at equilibrium is C_e (mg L^{-1}). q_e is considered as the adsorption capacity (mg g^{-1}), the volume of DI, TE and CEP solution is V(L) and the mass of ACB is defined by W(g).

Moreover, ACB (0.25 g) was loaded with 50 mL of high (400 mg L^{-1}) and low concentrations (0.1 mg L^{-1}) of DI, TE, and CEP in two matrices (MQ or AWW) in polyethylene tubes for 24 h on a roller shaker. The supernatant was collected by filter paper and oven-dried at 60 °C to obtain the ACB-LPs and were named ACB-LP(DI), ACB-LP(TE), and ACB-LP(CEP), respectively (Table 2). The prepared materials were used for the toxicity test.

Range-finding toxicity tests were conducted to select suitable doses of ACB and ACB-LPs (results presented in the supplementary material). In selecting the concentration ranges for the experiments, a combination of factors, such as typical environmental concentrations detected in wastewater treatment effluents and concentrations reported in literature, were considered (Yang et al., 2017; Juela, 2022; Priya et al., 2022). The selected dosages were examined to preferably obtain no observable effect at the lowest dose and 100% effect at the highest dose on *D. magna* (OECD, 2004). Finally, the following doses of ACB and ACB-LP; 0.0125, 0.025, 0.05, 0.1 and 0.2 g L^{-1} in AFW were used in the definitive toxicity test.

The toxicity of different concentrations (10, 20, 40, 80, and 160 mg L^{-1}) of DI, TE, and CEP in artificial freshwater (AFW) was examined to provide positive control to compare the results of this study with findings of earlier toxicity tests carried out on pharmaceuticals (Table 2).

Table 2
Characteristics of different treatments, preparation (matrix and material) and concentrations of the materials used in the AFW.

Treatments	Preparation Matrix and material used for loading			Concentration of the material in AFW	
ACB	Matrix			Concentration of ACB in AFW	
	MQ		AWW		
ACB	x		x	0.0125, 0.025, 0.05, 0.1 and 0.2 g L^{-1}	
ACB-LP	Matrix		Pharmaceutical concentration	Concentration of ACB-LP in AFW	
	MQ	AWW	Low (0.1 mg L^{-1})	High (400 mg L^{-1})	0.0125, 0.025, 0.05, 0.1 and 0.2 g L^{-1}
ACB-LP(DI)	x		x		
	x			x	
		x	x		
		x		x	
ACB-LP(TE)	x		x		
	x			x	
		x	x		
		x		x	
ACB-LP(CEP)	x		x		
	x			x	
		x	x		
		x		x	
Pharmaceuticals	Matrix			Concentration of pharmaceuticals in AFW	
	MQ		AWW	10, 20, 40, 80 and 160 mg L^{-1}	
DI	x				
TE	x				
CEP	x				

In this table, "x" indicates the presence of the treatment, preparation, or pharmaceutical in the respective matrix.

2.2.2. Acute toxicity test

The test conditions were similar to the incubation period despite the fact that a different photoperiod regime was used for all the treatments (16 h light and 8 h dark) except for TE. TE treatments were wrapped in aluminum foil to provide darkness and avoid degradation of the pharmaceuticals. The neonates were transferred to 50 mL cylindrical vials which contained 20 mL of the media and either of the ACB, ACB-LP or the studied pharmaceuticals. Five neonates were transferred to every replicate (n = 4) in each treatment (the total number of neonates for each treatment was 20). To test the validity of the experiment, proper control treatments (≥90% survival rate) with sufficient dissolved oxygen level (≥3 mg L^{-1}) were established (OECD, 2004). The number of immobilized neonates was monitored and recorded, after 24 and 48 h. To further observe the possible ingestion and effects of ACB and ACB-LP on *D. magna*, the organisms were microscopically analyzed (n = 1–3 per treatment) at 3.2X magnification, using Stereomicroscope Zeiss stemi 508, Germany (Schneider et al., 2012).

2.3. Analyses of water samples

Analyses of the pharmaceuticals in water samples were performed by liquid chromatograph (LC) followed by electrospray ionization triple quadrupole mass spectrometer (MS) (Shimadzu Nexera X2 UHPLC followed by Shimadzu LCMS-8040, Japan). A Kinetex C18 column (1.7 μm, 100 × 3 mm, Phenomenex Inc., USA) was used for LC separations.

The mobile phases used were water with 0.1 % formic acid and acetonitrile. The flow rate was 0.4 mL min^{-1}. The column oven was set to 30 °C. The initial condition, including 95% water and 5% acetonitrile, was maintained for 2 min before changing to 10% water and 90% acetonitrile following a 10-min gradient. These conditions were followed by 3 min equilibration. Therefore, the total run was 15 min. Detection was performed in positive multiple reaction monitoring (MRM) mode. The following MRM transitions of m/z 296 - > m/z 214, m/z 445 - > m/z 410 and m/z 348 - > m/z 158 were used to quantify DI, TE, and CEP, respectively.

The water quality, including pH (Mettler Toledo AG, Switzerland), oxygen content (Hach HQ40d, USA), conductivity (Radiometer Analytical S.A., France), and temperature (Hach HQ40d, USA) were measured at the initial and final stage of the toxicity tests.

2.4. Data analyses

The data analyses included recording the immobility as the endpoint for toxicity and calculation of EC_{50} values. Immobility rate was calculated as the total number of immobile animals divided by total number of animals in one treatment multiplied by 100. GraphPad Prism (version 5.0) for Windows (GraphPad Software, San Diego California USA) was used to plot immobility rate against the log transformed administered doses, using the dose-response nonlinear regression analyses. Moreover, this software was used to perform the EC_{50} and statistical analyses of the immobility rates among the treatments. The difference was considered significant when *P*-value $\leq$0.05.

3. Results

3.1. Removal efficiency of ACB

The initial pharmaceutical concentration (0.001, 0.005, 0.025, 0.05, 0.1 mg L^{-1}) dissolved in MQ influenced the adsorption efficiency of ACB (Fig. 1(a, b, and c)). By increasing the pharmaceutical concentration, the adsorption of DI, TE and CEP increased. For DI, the removal efficiency showed a constant increasing trend from the lowest (30.7% for 0.001 mg L^{-1}) to the highest (97.5% for 0.1 mg L^{-1}) pharmaceutical concentration. However, saturation of the adsorption sites for DI and TE occurred after increasing the initial concentration of the pharmaceutical to 0.025 mg L^{-1}. For CEP, the saturation of the adsorption sites was achieved at lower concentrations (98.4% for 0.005 mg L^{-1}). The adsorption capacity of ACB for removal of DI, TE, and CEP at the highest initial concentration was 0.998, 0.925, and 0.974 mg g^{-1}, respectively.

Moreover, different initial pharmaceutical concentrations (10, 20, and 40 mg L^{-1}) in AWW influenced the adsorption efficiency of ACB (Fig. 1(d, e, and f)). The results revealed that the removal percentage of ACB decreased as the initial concentration of the pharmaceuticals in AWW increased from 10 to 40 mg L^{-1}. For instance, the removal efficiency of ACB for DI declined from 100% to 50% after increasing the initial pharmaceutical concentration from 10 to 40 mg L^{-1}. A higher concentration of TE revealed a similar trend; for instance, removal efficiency of ACB for TE was 75% and 21% when the starting concentration of TE was 10 and 40 mg L^{-1}, respectively. In the case of CEP, the removal percentage of ACB decreased from 55% to 16% after increasing the initial concentration of CEP from 10 to 40 mg L^{-1}.

3.2. Ecotoxicity studies

3.2.1. Environmental conditions and water quality parameters

In this study, the measured water chemical properties were pH, oxygen content, conductivity, and temperature. The water quality parameters were relatively constant during the experiment (Table 3).

The pH was circumneutral, ranging from 7.3 to 7.8 across different treatments. The oxygen content was stable (over 95%) and sufficient for the survival of *D. magna* (OECD, 2004). The conductivity of the media varied from 591 to 695 μS cm^{-1} among the studied treatments. The average temperature was 21 °C.

3.2.2. Microscopic analyses

The microscopic analyses of the *D. magna* exposed to different concentrations of ACB and ACB-LP (loaded with high concentration of pharmaceuticals in MQ and AWW) revealed interesting observations (Figs. 2 and 3). The immobilization rate corresponded with the amount of ACB and ACB-LP (loaded with high concentration of pharmaceuticals in MQ) that either bound to the body surface or found in the gut of *D. magna* (Fig. 2). At 0.0125 g L^{-1}, there were no visible morphological

Table 3
Water chemical properties of the media.

Water properties	pH		Oxygen content (%)		Conductivity (μS cm^{-1})		Temperature (°C)	
	pH_i[a]	pH_f[b]	O_i[a]	O_f[b]	C_i[a]	C_f[b]	T_i[a]	T_f[b]
ACB	7.6	7.8	96.0	95.6	672.4	658.8	20.5	21.0
DI	7.7	7.8	96.2	96.5	634.0	679.6	21.0	21.5
TE	7.6	7.4	96.3	97.5	619.8	591.0	20.0	20.5
CEP	7.5	7.5	96.1	97.5	695.0	642.0	21.0	21.5
ACB-LP(DI)	7.6	7.8	96.6	95.4	680.0	628.0	21.0	21.5
ACB-LP(TE)	7.4	7.3	96.0	97.5	690.0	627.0	21.0	21.5
ACB-LP(CEP)	7.4	7.7	96.1	97.0	695.0	627.2	21.0	21.5

[a] Initial pH.
[b] Final pH.

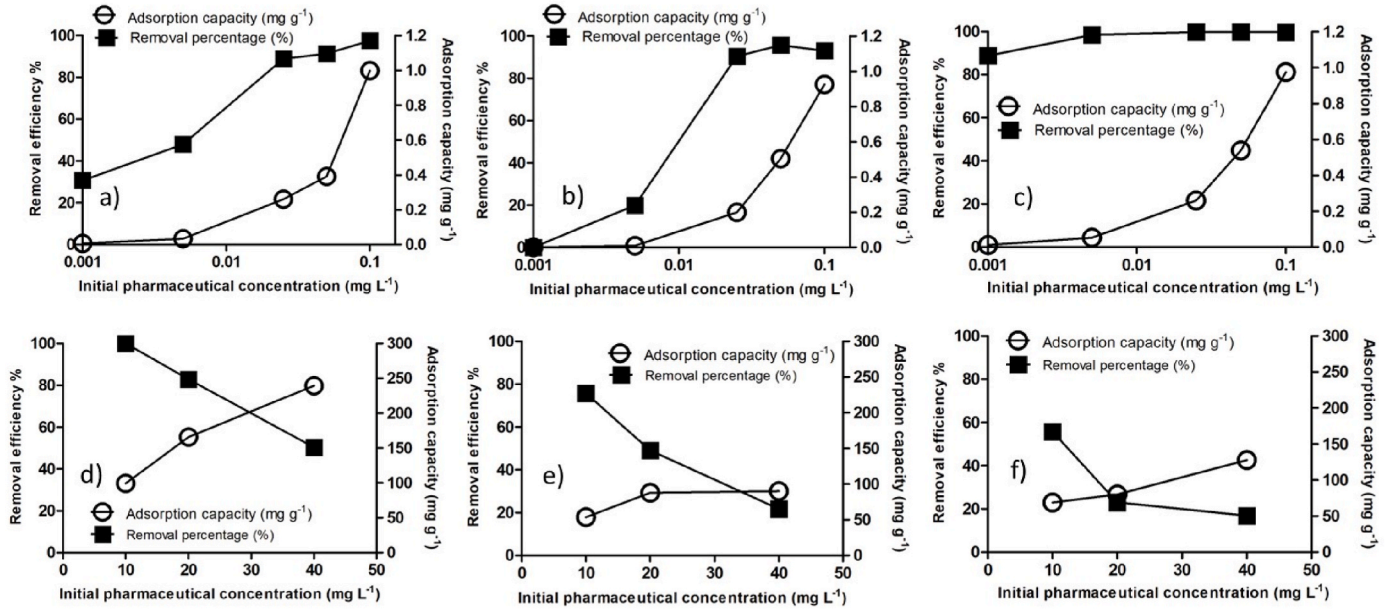

Fig. 1. Effect of initial pharmaceutical concentration in MQ on (a) DI,(b) TE, and (c) CEP removal by ACB (at initial concentration values of 0.001, 0.005, 0.025, 0.05, and 0.1 mg L^{-1}) and effect of initial pharmaceutical concentration in AWW on (d) DI,(e) TE, and (f) CEP removal by ACB (at initial concentration values of 10, 20 and 40 mg L^{-1}, adsorbent dose: 0.1 g L^{-1}, agitation speed: 80 rpm for 24 h, temperature: 25 ± 1 °C, n = 2, SD < ±5%).

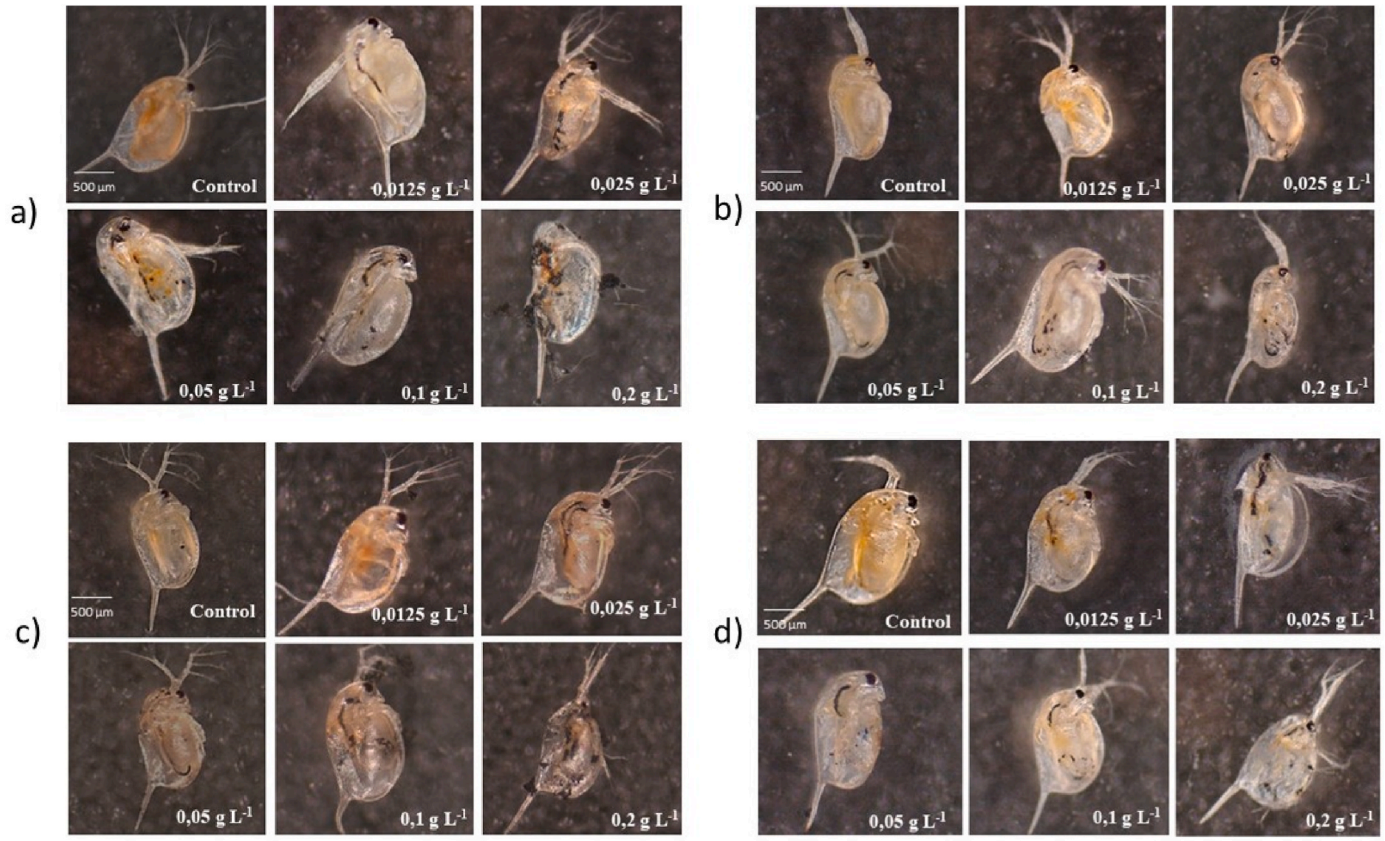

Fig. 2. Images of *D. magna* from acute tests exposed to different concentrations of a) ACB, b) ACB-LP(DI) c) ACB-LP(TE), and d) ACB-LP(CEP) loaded with a high concentration (400 mg L^{-1}) of pharmaceuticals in MQ. Exposure period: 48 h. Each panel includes the negative control for comparison within the same batch.

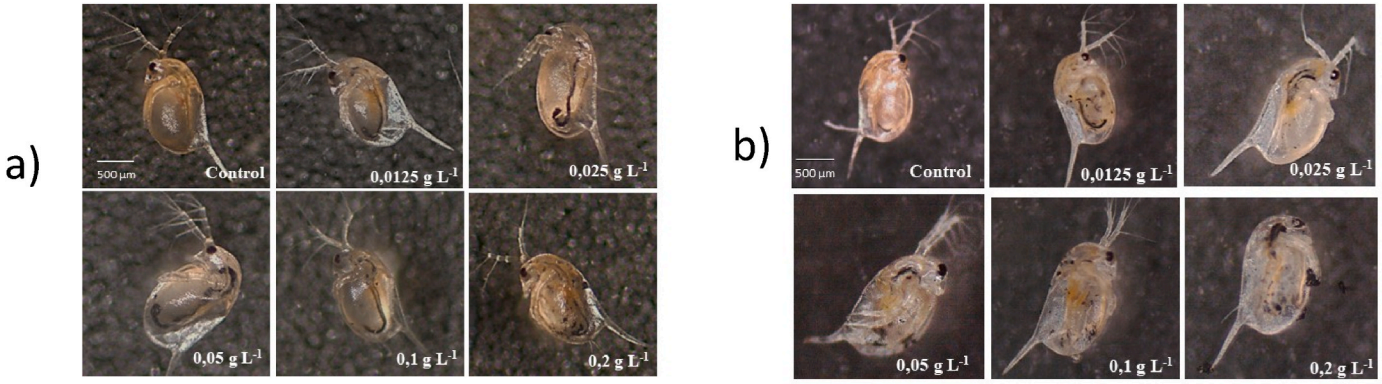

Fig. 3. Images of *D. magna* from acute test exposed to different concentrations of a) ACB-LP(DI) and b) ACB-LP(CEP) loaded with a high concentration (400 mg L^{-1}) of pharmaceuticals in AWW. Exposure period: 48 h. Each panel includes the negative control for comparison within the same batch.

alterations compared to the control treatment, with the exception of ACB and ACB-LP(CEP). After increasing the concentration of ACB and ACB-LPs to 0.025 g L^{-1}, the particles were partially attached to different body structures, such as antennas. By increasing the concentration of ACB and ACB-LPs to 0.05 and 0.1 g L^{-1}, more aggregation of the particles on the body surface and the intestine became visible. At the highest tested concentration of 0.2 g L^{-1}, *D. magna* was thoroughly covered with ACB and ACB-LPs particles. These particles were not only observed in the intestine of the *D. magna*, but also aggregated to the abdomen and antenna.

Microscopic analyses showed that ACB-LP particles loaded in AWW appear to be less adherent to the antenna and to the body of *D. magna* (Fig. 3). Despite this reduced adhesion, the presence of the particles was visible in the entire gut system even at the lowest concentrations (0.0125 and 0.025 g L^{-1}). Adhesion of the particles to the body of *D. magna* became obvious after increasing the concentration to 0.2 g L^{-1}.

3.2.3. Ecotoxicity of pharmaceuticals, ACB, and ACB-LPs

To compare the effect of pharmaceuticals and the adsorbents (ACB and ACB-LP) on *D. magna,* the effect of different concentrations of pharmaceuticals were plotted (Fig. 4).

After 48 h, TE and CEP did not cause any significant toxicity compared to controls at any of the tested concentrations. However, after exposure to the highest concentrations of DI, 100% of the daphnids were observed to be immobilized.

The EC_{50} after 48 h for ACB loaded in MQ and AWW was 52.1 and

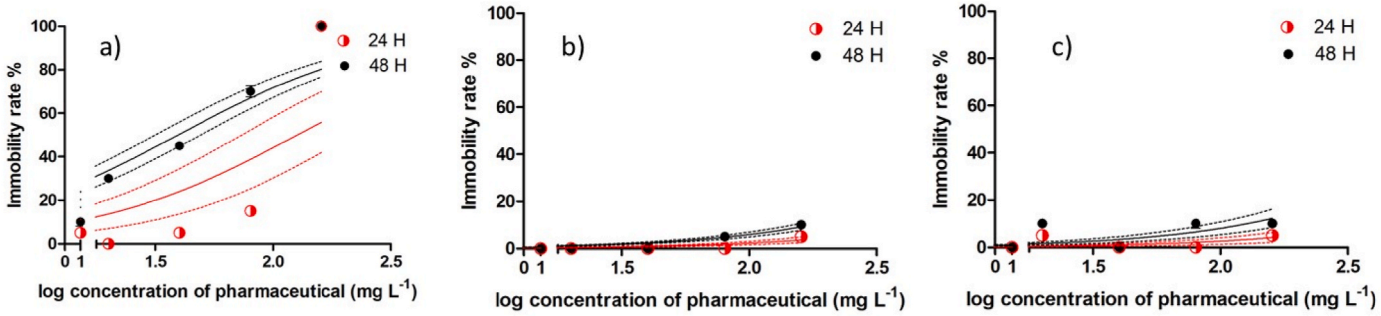

Fig. 4. Immobility% of *D. magna* exposed to different concentrations of pharmaceuticals (10, 20, 40, 80 and 160 mg L^{-1}); a) DI, b) TE and c) CEP after 24 (red) and 48 (black) hours of exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

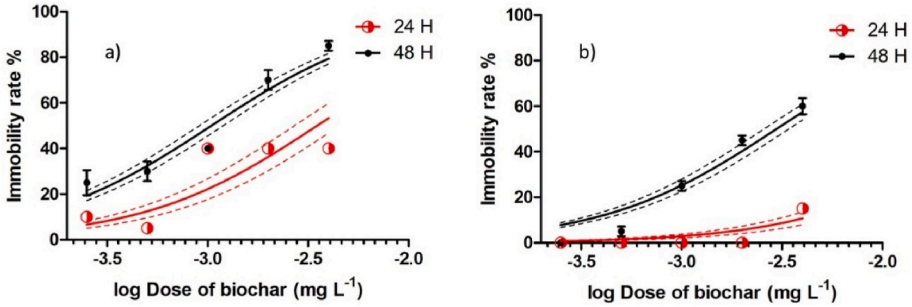

Fig. 5. Immobility% of *D. magna* exposed to different concentrations (0.0125, 0.025, 0.05, 0.1 and 0.2 g L^{-1}) of a) ACB and b) ACB loaded by AWW after 24 (red) and 48 (black) hours of exposure. Dash lines are the corresponding 95% upper and lower confidence bands and circles represent the mean of the immobility at each dose (number of organisms = 20), this info is similar in Figure 6 and 7). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

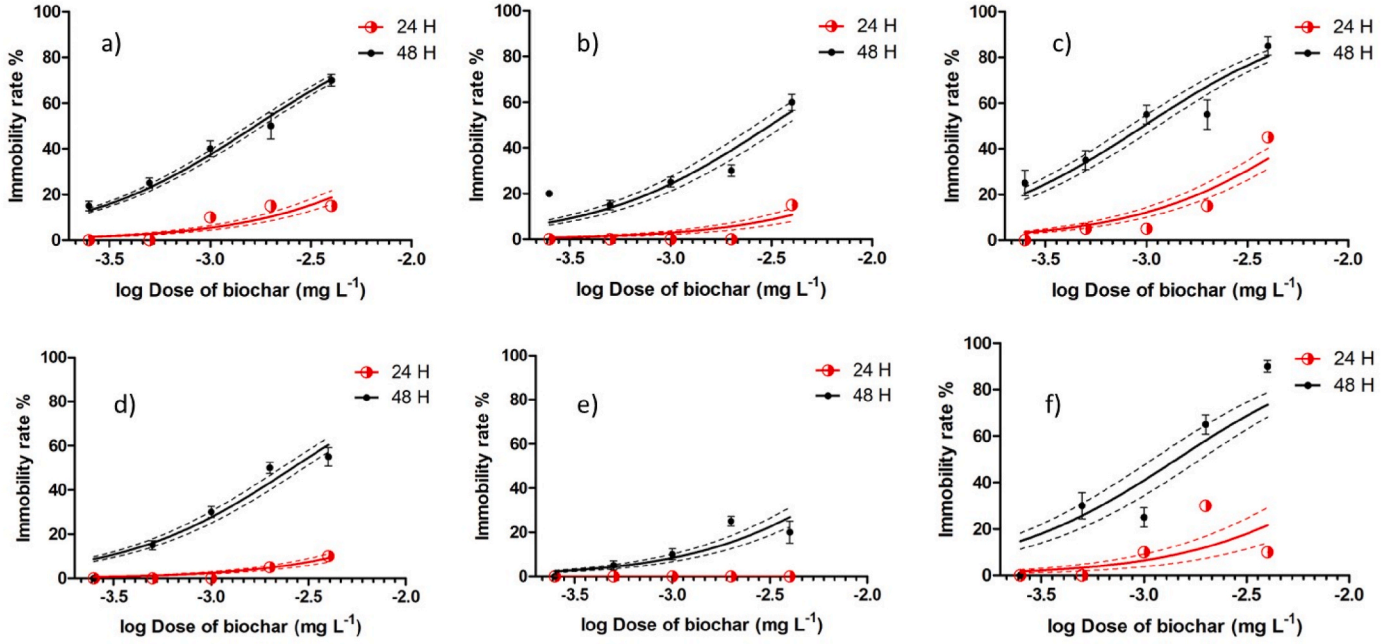

Fig. 6. Immobility% of *D. magna* exposed to ACB loaded by low concentration of pharmaceuticals (0.1 mg L^{-1}) in MQ; a) ACB-LP(DI), b) ACB-LP(TE), and c) ACB-LP (CEP) and percentage of immobility rate of *D. magna* exposed to ACB loaded by low concentration of pharmaceuticals (0.1 mg L^{-1}) in AWW; e) ACB-LP(DI), f) ACB-LP (TE), and g) ACB-LP(CEP) after 24 (red) and 48 (black) hours of exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

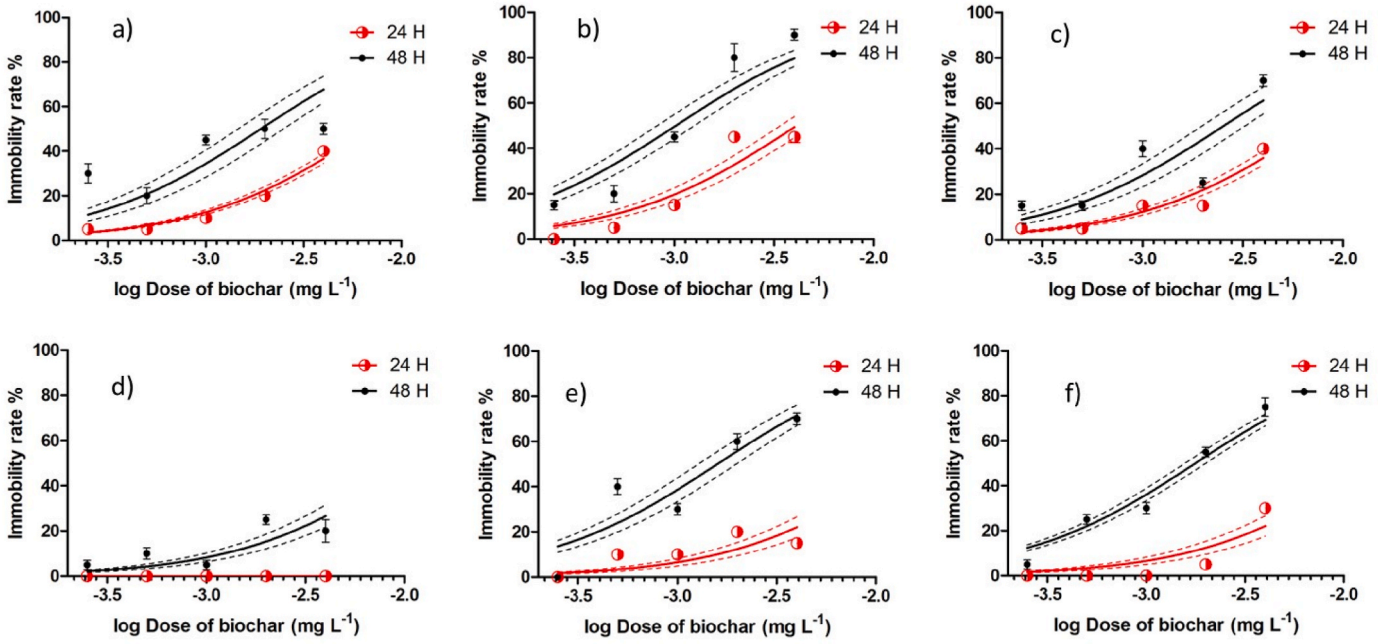

Fig. 7. Immobility% of *D. magna* exposed to ACB loaded by high concentration of pharmaceuticals (400 mg L^{-1}) in MQ; a) ACB-LP(DI), b) ACB-LP(TE), and c) ACB-LP(CEP) and percentage of immobility rate of *D. magna* exposed to ACB loaded by high concentration of pharmaceuticals (400 mg L^{-1}) in AWW; d) ACB-LP(DI), e) ACB-LP(TE), and f) ACB-LP(CEP) after 24 (red) and 48 (black) hours of exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
EC_{50} of ACB-LP in high and low concentrations of pharmaceuticals with corresponding 95% confidence intervals.

Material	400 mg L^{-1} Loaded in MQ	400 mg L^{-1} Loaded in AWW	0.1 mg L^{-1} Loaded in MQ	0.1 mg L^{-1} Loaded in AWW
ACB-LP (DI)	147.0 (132.8–185.1)	545 (436.5–680.5)	83.7 (76.9–91.2)	131.1 (114.6–149.8)
ACB-LP (TE)	50.9 (41.0–63.2)	79.3 (63.9–99.1)	156.7 (132.1–185.9)	437.4 (385.8–495.9)
ACB-LP (CEP)	130.6 (98.5–162.3)	88.5 (78.4–99.9)	48.5 (41.3–56.9)	72.1 (54.9–94.7)

148.2 mg L^{-1}, respectively. The EC_{50} of ACB-LP loaded in different matrices revealed both higher and lower values compared to ACB (Table 4). However, the highest EC_{50} value was 545 mg L^{-1} for ACB-LP (DI) loaded in 400 mg L^{-1} of DI in AWW. The higher EC_{50} values were mostly observed in ACB-LPs loaded in AWW. The lowest EC_{50} value was 48.5 mg L^{-1} for ACB-LP(CEP) loaded in 0.1 mg L^{-1} in MQ.

Throughout the exposure period the observed mortality rate of the *D. magna* in the control groups was less than 5%, indicating the acceptability of the data. For all ACBs and ACB-LPs the survival rate of the daphnids at the lowest dose (0.0125 g L^{-1}) was higher than 90%, while this value decreased considerably at higher doses.

The daphnids exposed to ACB (positive control) exhibited an immobility rate of 85% after 48 h, yielding an EC_{50} value of 52.1 mg L^{-1} at the highest exposure concentration, with upper and lower concentration limits of 45.4 and 59.8 mg L^{-1}, respectively (Fig. 5). The loading of ACB in AWW decreased the immobility rate of the positive control to 60% and increased the EC_{50} to 148.2 mg L^{-1}, with upper and lower concentration limits of 128.5 and 171 mg L^{-1}, respectively. As a result, the ACB loaded in AWW (Fig. 5b) was significantly less toxic than the ACB loaded in MQ (Fig. 5a) after 24 and 48 h ($F_{1,48} = 110.7$, $P < 0.0001$ and $F_{1,48} = 128.8$, $P < 0.0001$, respectively).

After loading ACB in low doses of pharmaceuticals (0.1 mg L^{-1}) in MQ, the immobility rate of *D. magna* was the highest (85%) for ACB-LP (CEP) at the highest concentration (0.2 g L^{-1}) with the EC_{50} value of 48.5 mg L^{-1} followed by ACB-LP(DI) and ACB-LP(TE) with immobility rates of 70% and 60% after 48 h, and EC_{50} values of 83.7 and 156.7 mg L^{-1}, respectively (Fig. 6 (a, b, and c).

A similar trend was obvious after loading ACB by low doses of pharmaceuticals (0.1 mg L^{-1}) in AWW. The highest immobility rate of *D. magna* after 48 h was for ACB-LP(CEP), (90%) with EC_{50} of 72.1 mg L^{-1} followed by ACB-LP(DI), (55%) and ACB-LP(TE), (30%) with EC_{50} of 131.1 and 437.4 mg L^{-1}, respectively (Fig. 6 (d, e, and f).

After loading ACB in high doses of pharmaceuticals (400 mg L^{-1}) in MQ (Fig. 7 (a, b, and c) and AWW (Fig. 7 (d, e, and f), the highest immobility rate was observed for ACB-LP(TE) (90% in MQ and 70% in AWW) with EC_{50} values of 50.9 and 79.3 mg L^{-1}, respectively. At these high concentrations, the lowest immobility rate was detected for ACB-LP (DI) in MQ (50%) and AWW (20%) with EC_{50} of 95.4 and 545 mg L^{-1}, respectively.

4. Discussion

The current study aimed to evaluate the potential ecotoxic effects of biomass-based adsorbent materials, utilized in wastewater treatment on *D. magna*. ACB, capable of efficiently removing high concentrations of various pharmaceuticals, has practical potential for adsorbing pharmaceuticals from wastewater. In real-life scenarios, pharmaceuticals are usually observed at low concentrations and are often mixed with other contaminants in wastewater. Thus, preliminary experiments were conducted to investigate how low initial pharmaceutical concentrations and the coexistence of other wastewater constituents (e.g., urea) influence the removal efficiency of ACBs. Additionally, the possible effects of ACB, ACB-LPs and pharmaceuticals on *D. magna* were investigated.

4.1. Removal efficiency of ACB

At the low initial pharmaceutical concentration, an increase in the starting concentration increased both the adsorption capacity and the removal percentage, consistent with findings of Sekulic et al. (2019), on

sulfamethoxazole removal at low concentrations. However, ACB demonstrated higher removal efficiency in our previous study with elevated initial pharmaceutical concentrations in MQ (Shirani et al., 2020). Reduced removal efficiency caused by lower adsorbate concentration can be due to weaker adsorbent-adsorbate interactions, as supported by FTIR data (Kyzas et al., 2013). At lower concentrations, mass transfer resistance could potentially prevent the adsorbent-adsorbate collisions. At higher concentrations, increasing the driving force can resolve this (Zhang et al., 2014; Shayesteh et al., 2020). Comparison of the data from this study and our previous study shows a lower removal efficiency of ACB in AWW compared to MQ (Shirani et al., 2020). For instance, with an initial concentration of 40 mg L^{-1} for DI, TE, and CEP dissolved in MQ, the average removal efficiency of ACB was 53%, 78%, and 87%, respectively. However, in AWW, it decreased to 50%, 21%, and 36%, respectively. The decrease in removal efficiency of ACB in AWW could be attributed to the interaction between the wastewater constituents and the pharmaceuticals hindering their adsorption by formation of wastewater constituents-pharmaceuticals complexes (Schmidt et al., 2024). Moreover, Table 3 shows the pH of the media at approximately 7.5, which was higher than the pH of MQ measured in our previous study (Shirani et al., 2020). At pH 7.5, DI and CEP are negatively charged, and TE is a zwitterion (Table 1). The zero point of charge (pH_{zpc}) of ACB was determined to be 5.12. At pH values higher than this threshold the ACB acquires a negative charge (Shirani et al., 2020). Thus, electrostatic repulsion between the adsorbate and the adsorbent with similar charges may have led to lower removal efficiency (Guillossou et al., 2020; Oliveira et al., 2018). Another factor can be due to occupation of ACB's adsorption sites by the other constituents of AWW. The components of AWW compete with the adsorbate for the adsorbent's available sites, blocking them and reducing adsorption efficiency in comparison to ultra-pure water matrices (Guillossou et al., 2020). Similar results were observed in an earlier study in which the presence of urea displayed a negative effect on adsorption of tetracycline (Li and Shi, 2022). In human urine the available form of nitrogen is ammonia (NH^{4+}) while phosphate is present in the form of HPO_4^{2-} and $H_2PO_4^-$ (Masrura et al., 2021). In the studied conditions ACB carries a negative surface charge which is favorable for the adsorption of ammonia and potentially not affecting the adsorption of phosphate. Furthermore, the presence of divalent cations such as Mg^{2+} and Ca^{2+} in wastewater may have inhibited the adsorption of the pharmaceuticals through altering the pH, increasing the ionic strength, forming complexes and inducing competitive adsorption on the surface of the adsorbent (Guan et al., 2022).

4.2. Microscopic analyses

The imagery analyses revealed that, after increasing ACB and ACB-LPs concentrations (loaded by high concentrations of pharmaceuticals in MQ) from 0.0125 to 0.025 g L^{-1}, the particles were partially attached to different body structures (Fig. 2) due to electrostatic and hydrophobic interactions between functional groups of the adsorbents (ACB and ACB-LPs) and the *D. magna.* Other researchers have reported similar results, using microplastics (Yin et al., 2020). The acute toxicity test images demonstrated that ACB and ACB-LP (loaded by high concentrations of pharmaceuticals in MQ) adhere to the gills and cuticles of the *D. magna* (Fig. 2). This may inhibit the swimming movements and respiration of the aquatic organisms, causing immobilization and death (Woermann and Sures, 2020). In comparison, ACB-LPs (loaded by high concentrations of pharmaceuticals in AWW) did not significantly adhere to the cuticles of *D. magna* in different doses except for ACB-LP(CEP) and thus, did not cause impairment (Fig. 3). This can be attributed to the complete occupation of the adsorbent's binding sites by the AWW components, leading to no free-binding sites to allow binding to the cuticles of *D. magna* (Woermann and Sures, 2020). The non-availability of the binding sites of the adsorbent may have another positive impact on the survival of *D. magna* as the adsorbent would be unable to bind with the components from the test media (Woermann and Sures, 2020). Moreover, desorption of AWW components from the adsorbent's surface is an important factor to consider. Components that are physically adsorbed to biochar can be efficiently desorbed (F. Zhang et al., 2020a). Desorbed Ca^{2+} and Mg^{2+} may enter the test media. In an earlier study the increase of water hardness as a matter of increase in these two cations led to protection of *D. magna* against chronic Ni toxicity (Deleebeeck et al., 2008). This may be another reason why ACB-LPs loaded in AWW might be less harmful compared to those loaded in MQ. ACB and ACB-LP mostly precipitated at the bottom of the apparatus and in some vials, they could be observed floating in the middle and top part of the experimental vials. Observations from the experiments suggested possible preference of these areas by the daphnids, which might lead to higher ingestion of ACB and ACB-LP. At higher concentrations, ACB and ACB-LP were present in the gut system of *D. magna*, implying ingestion and a possible higher mortality rate among those organisms (Figs. 2 and 3). In a previous study, toxicity of activated carbon on *D. magna* was attributed to chemical (modification of the solution's chemistry through release of chemicals associated with activated carbon) and/or physical basis (obstruction of the digestive system and movement restriction), and adsorption of the essential skin compartments by activated carbon (Jonker et al., 2009). Additionally, while the focus of this study was not on the long-term effects of the adsorbents, future research in this area is important. Previous studies have shown that bulk biochar can disintegrate into smaller particles such as biochar nanoparticles, which can adhere to *Daphnia magna* over time (Z. Liu et al., 2022b). This suggests that long-term particle adherence may lead to bioaccumulation and trophic transfer within aquatic environments and thus have an impact on growth, reproduction, and overall ecosystem health (Huang et al., 2021; Z. Liu et al., 2022b).

4.3. Comparison of ecotoxicity of different pharmaceuticals

In the literature, the EC_{50} values for *D. magna* exposed to DI, TE, and CEP ranged from 23 to 68, 198–617, and 37–2268 mg L^{-1}, respectively while, the EC_{50} values gained from this study were 39.4, 586.4, and 1439.5 mg L^{-1}, respectively (Ferrari et al., 2003; Li, 2013; Daghrir and Drogui, 2013; SÖNMEZ and SİVRİ, 2020; Białk-Bielińska et al., 2022; Guler and Solmaz, 2022; Hu et al., 2022). In case of TE, the high resistance of daphnids and thus, higher EC_{50} may be attributed to the sorptive behavior of TE towards components of the medium and as a result a decrease in its concentration in the growth medium to half of the nominal concentration after 2 h (Martin, 1979). TE has a high tendency to complex with heavy metals such as Ca^{2+} and Mg^{2+} that are present in the culture medium of *D. magna* (Kim et al., 2014). One molecule of Ca^{2+} and Mg^{2+} causes complexation with one and two TE molecules, respectively (Jin et al., 2007), forming various degradation products which may affect its toxicity compared to the parent compound (Pulicharla et al., 2017). These metal complexes change the pharmacological and toxicological properties of many drugs (Anacona and Rodriguez, 2004).

DI exhibits low affinity for Ca^{2+} and Mg^{2+} (Bui and Choi, 2010). Unlike TE, which demonstrated significant decrease (half of the nominal concentration) after 2 h (Martin, 1979), DI concentration in the medium remained relatively stable after 48 h (Nkoom et al., 2019). This may have caused higher mortality rates correlated with exposure to high DI concentrations. Previous study reveals that CEP alone is much less toxic (LC_{50} value of 2268 mg L^{-1}) to *D. magna* in comparison to its toxicity in the presence of wastewater components which could be due to the combined toxicity of several substances (Xue et al., 2022). In aquatic ecosystems, the presence of multiple pollutants and environmental variables can affect toxicity differently than what has been predicted by individual substance assessments (Xue et al., 2022). Our study demonstrates that the combination of pharmaceuticals and adsorbents may cause synergistic or antagonistic effects on aquatic organisms. The results indicated that the acute toxicity of TE and CEP in *D. magna* was

increased after ACB was loaded by the pharmaceuticals, while DF showed antagonistic effects at different concentrations. Similar synergistic effects were obtained by other studies revealing that phenicol antibiotics have a higher acute toxicity in *D. magna* when combined by suspended particles due to potential cellular oxidative damage (Y. Zhang et al., 2021b). Moreover, both synergistic and antagonistic effects were observed after *D. magna* were exposed to both antibiotics and carbon nanotubes (Simon et al., 2015; Zhang et al., 2023). Furthermore, practical applications of adsorbents such as ACB in water treatment face challenges in controlling the compounds captured due to the variability of contaminants in wastewater. To evaluate this effect, we focused on evaluating the performance of ACB loaded with pharmaceuticals, rather than solely assessing the ecotoxicity of the pharmaceuticals.

4.4. Comparison of ecotoxicity of ACB with ACB-LP

According to EU Directive 93/67/EEC, based on the values of EC_{50} obtained from the 48 h *D. magna* tests, compounds with EC_{50} lower than 1 mg L^{-1} are categorized as very toxic, while substances with EC_{50} in the range of 1–10 mg L^{-1} and 10–100 mg L^{-1} are classified as toxic and harmful to the aquatic environment, respectively (Cec, 1996).

Results from the acute toxicity tests reveal that the ACB loaded in AWW and ACB-LP have lower risk for *D. magna* compared to the ACB, as evidenced by the EC_{50} value of ACB being 52.1 mg L^{-1}. The EC_{50} of other loaded material was significantly higher, except for ACB loaded by low concentration of CEP in MQ (48.5 mg L^{-1}). This may be attributed to the presence of toxic compounds such as dioxins and polycyclic aromatic hydrocarbons on the biochar surface, depending on the production process (Hale et al., 2012). Another study reported that activated biochar alone had higher toxicity to *D. magna,* while it had lower toxicity in the presence of copper (Shim et al., 2015). Toxicity of ACB could also be due to the presence of environmentally persistent free radicals (EPFRs), which cause oxidative stress in aquatic organisms (Zhang et al., 2019). Increasing the concentration of biochar directly effects the enhancement of the EPFRs in water (K. Liu et al., 2022a). However, after loading of the biochar with pharmaceuticals and preparation of ACB-LP, the effect of EPFR may be inhibited and thus cause lower toxicity towards the daphnids. Although, in this study there were no direct measurements of the EPFRs, their potential decrease after adsorption is concluded from previous studies and literature on similar systems (Huang et al., 2022; Y. Zhang et al., 2020b). Moreover, after activation, sharp edges appeared on the surface of ACB (Shirani et al., 2020; X. Zhang et al., 2021a). This might have caused the higher mortality rate in the *D. magna* due to ingestion or cuticle damage. However, after adsorption (ACB-LP), the pores of the biochar become clogged by the adsorbate, leading to a smoother surface area that may be less harmful to the crustaceans. ACB contains numerous vacant pores that can adsorb available nutrients in the medium of *D. magna*, causing nutrient deficiency and subsequently leading to higher mortality rates (Woermann and Sures, 2020).

4.5. Comparison of ecotoxicity of different ACB-LPs

After exposing *D. magna* to ACB-LP loaded by low concentrations of pharmaceuticals in both MQ and AWW, the highest and lowest mortality was recorded for ACB-LP(CEP) and ACB-LP(TE), respectively. As mentioned earlier, removal efficiency of ACB for CEP decreased considerably after changing the matrix from MQ to AWW. This significant reduction might be attributed to the presence of organics (e.g., beef extract), inorganics (e.g., sulfates, and bi-phosphate), and cations (e.g., Na^{+}, Mg^{2+}, K^{+}, and Ca^{2+}) in AWW, that have affinity for CEP and might have competed for the adsorption sites (Ahmed et al., 2017, 2018; Acelas et al., 2021). Moreover, cephalosporins, including CEP, have low stability in wastewater due to the presence of β-lactam in their structure (Moghaddam et al., 2023). Another study reported that natural degradation of CEP may occur in wastewater before treatment (Al-Gheethi et al., 2017). Consequently, the concentration of CEP adsorbed on the surface of the ACB-LP(CEP) was reduced in AWW than in MQ, which might have led to the mere presence of EPFRs and components of AWW on the surface of ACB-LP(CEP), leading to higher toxicity.

On the other hand, TE can bind with cations such as, Ca^{2+} and Mg^{2+} and with sludge or sediments particles to form a ternary complex with higher stability (Xu et al., 2021). At low concentration, ACB-LP(TE) may have formed stable complexes with the cations present in AWW, reducing its effect on the aquatic organisms. Conversely, when ACB was loaded in higher concentrations of TE in both matrices (AWW and MQ) the lowest EC_{50} among the other studied pharmaceuticals was observed. This could be attributed to the saturation of the sites with ternary complex, while the excess TE have remained unchanged on the surface of ACB-LP(TE). Moreover, the EPFR on the surface of the biochar have the capability to degrade TE, leading to possible production of intermediates with higher toxicity (K. Liu et al., 2022b). A prior study indicated that biochar prepared at higher temperatures (500 °C), compared to lower temperatures (400 °C), results in the adsorption of TE in the internal small pores. This restricts its primary availability to organisms due to the small pore size of the biochar (Wang et al., 2020). In addition, water is not capable to efficiently mobilize TE from the pores of the biochar to the surface (Wang et al., 2020). Therefore, the external functional groups present on the surface of the ACB-LP(TE) were accessible for *D. magna* and attached to the body surface and caused immobility and finally mortality like ACB.

As a hydrophobic pharmaceutical, DI exhibits high tendency to be adsorbed on the surface of ACB-LP(DI) through strong π-π bonding, hydrophobic and electrostatic adsorption interactions (Shirani et al., 2020). These interactions facilitate bonding of the functional groups of DI with functional groups of ACB-LP(DI) and thus, leaves no extra functional groups to interact with *D. magna* and reduces the toxicity of each substance, individually. Strong interactions between the adsorbent and adsorbate such as π-π EDA can significantly prevent desorption (F. Zhang et al., 2020a). In another study, it was also noted that the sorption irreversibility in the adsorbent increases at elevated concentrations of the adsorbate (Martínez-Hernández et al., 2014). Therefore, these factors have restricted the release of DI into AFW, leading to lower toxicity after loading ACB-LP by high concentrations of pharmaceuticals in both MQ and AWW.

The EC_{50} of *D. magna* was lower with ACB-LP loaded by low and high concentrations of pharmaceuticals in MQ than in AWW, possibly due to the presence of other compounds in AWW. A previous study reported that the presence of adsorbable nutrients can alleviate the effects of pharmaceuticals on aquatic species (Aristi et al., 2016). The size of ACB particles after adsorption of different pharmaceuticals in AWW could also affect the toxicity, since large-sized particles are less ingestible by *D. magna*. Similar effects were observed in another study, showing higher mortality rate of *D. magna* upon ingestion of small-sized microplastic fragments compared to larger-sized beads (An et al., 2021). Moreover, it was reported that fine-sized fractions of activated carbon had stronger effects on the growth, reproduction, and lipid content of benthic organisms, even at low doses (0.05 and 0.25%), in contrast to large-sized particles (Nybom et al., 2012). However, the particle size was not measured in this study, which may be of interest for future investigation.

5. Limitations and future research directions

Adsorbents, specifically biochars, are prepared using various source materials and methods. Thus, they possess variable properties such as, adsorption efficiency and toxicity. This highlights the importance of conducting studies to optimize biochar's adsorption efficiency through in vitro studies as well as using various adsorbents to generalize our results. Moreover, the impact of environmental conditions, particle size, and the desorption of the adsorbed pharmaceuticals were not fully studied. In this case, molecular-scale studies, such as examining the oxidative stress of the organisms, would provide further clarity. This

study focused on acute toxicity tests of *D. magna* and the response of other aquatic species was not considered. Therefore, a broader chronic ecotoxicity assessment using various species such as, benthic organisms more exposed to settled adsorbents (Nybom et al., 2012), would be beneficial. Further research on the adsorbent's bioaccumulation potential and its interactions with other water treatment chemicals and real wastewater components (e.g. heavy metal, other pharmaceuticals and organics), leading to further synergistic and antagonistic effects, is also required. Finally, the practical implementation of using biochar-based adsorbents for wastewater treatment should be evaluated by determining the economic feasibility and scalability of these material. It is important to address these aspects in future studies to warrant the development of safer and more effective water treatment solutions.

6. Conclusion

Biochar-based materials have been widely studied for their potential as adsorbents in wastewater treatment. This necessitates further consideration of their adsorption efficiency in wastewater-relevant concentrations of pharmaceuticals and their removal potency in the presence of other wastewater constituents. In this study, the initial adsorbate concentration, mass transfer resistance, and competition from other wastewater components played an important role in ACB's removal efficiency. The results revealed that ACBs cause high mortality (85%) due to their chemical structure and the free-binding sites that can readily attach to cuticles of daphnids. Interestingly, ACB-LPs were mostly found to be less toxic than ACB. This may be attributed to the presence of various compounds in the artificial wastewater of which components are adsorbed onto the surface of ACB-LPs, blocking the pores and preventing the release of toxins. This study demonstrated the need to consider the environmental risk of adsorbent materials used in wastewater treatment. The need is further emphasized since an increase in the use of adsorbents is expected in the near future.

CRediT authorship contribution statement

Zahra Shirani: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Victor Carrasco-Navarro:** Writing – review & editing, Supervision, Resources, Methodology. **Soroush Majlesi:** Writing – review & editing, Investigation. **Pasi Yli-Pirilä:** Writing – review & editing, Investigation. **Jussi V. K. Kukkonen:** Writing – review & editing, Resources, Methodology, Conceptualization. **Jarkko Akkanen:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2024.123224.

Data availability

No data was used for the research described in the article.

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