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Approaches on phenoxy acid herbicides behavior in wastewater treatment plants and treatment relevance upon river biota

Diana Puiu [a,b,*], Corina Bradu [b], Costel Bumbac [a], Lucian Constantin [a], Lidia Kim [a], Olga Tiron [a], Carmen Postolache [b]

[a] National Research and Development Institute for Industrial Ecology, INCD ECOIND, 57-73 Drumul Podul Dambovitei, 060652 Bucharest, Romania
[b] Doctoral School in Ecology, University of Bucharest, 91-95 Splaiul Independenței, 050095 Bucharest, Romania

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ABSTRACT

The purpose of this study is to evaluate the risk contribution of wastewater treatment plants (WWTPs) to the contamination of receiving surface water with phenoxycarboxylic herbicides, focusing on the efficiency of WWTPs in removing these micropollutants. These compounds are widely used in agriculture and have moderate persistence, making them more likely to be present in the environment. However, there is a lack of significant studies regarding their presence in Romanian waters.

The concentration of phenoxycarboxylic acids in inflows and outflows ranged from below 3 ng/L to 247 ng/L. The efficiency of WWTPs showed a dynamic variability, beginning with a high removal rate of 4-chloro-2-methylphenoxyacetic acid (MCPA, 77.5%), followed by an accumulation of 2,4-dichlorophenoxyacetic acid (2,4-D) in the effluents, which increased by 247.5%. To move one step closer to solving the mechanistic puzzle, key factors affecting herbicide behavior in WWTPs were investigated, specifically hydraulic retention time and hydrolysis rate during the biological treatment phase. The comparison between estimated and measured results indicates that 2,4-D is removed slowly from WWTPs. Additionally, other compounds may be transformed from lower to higher hydrolyzable derivatives due to recirculation in biological treatment.

The ecotoxicological index indicates that both acute and chronic exposure to 2,4-D and MCPA may exert a *very high* risk to certain phytoplankton and macrophyte species. However, the grey water footprint of WWTP effluents contaminated with herbicides suggests a low overall impact, attributed to the high assimilation capacity of the Jiu and Danube rivers.

1. Introduction

Phenoxyherbicides are among the most intriguing pesticides because of their multiple action pathways: as pollutants at high doses, as weed control agents and defoliants at -optimal doses, and as plant growth regulators at low doses (Islam et al., 2018). However, the risk of pollution is the main concern of current studies, considering their moderate persistence in the environment and long-lasting toxic effects on aquatic organisms (ECHA, 2023) (Council, 2006) (Urbaniak and Mierzejewska, 2019). This concern is supported by evidence of herbicides occurring at high levels (μg/L or mg/L), or trace levels (ng/L) in relevant environmental compartments, especially near agricultural fields (Urbaniak and Mierzejewska, 2019) (Székács et al., 2015) (Gamhewage et al., 2019) (Ng et al., 2023) (Nam et al., 2014) (Lopes et al., 2020), as well as in unexpected locations such as Greenland ice sheets (Stibal et al., 2012) or Artic air particles (Balmer et al., 2019). In addition, herbicidal efficiency, good plant uptake, low production costs and the *grow now, clean up later* concept (Ekins and Zenghelis, 2021), suggest excessive and inappropriate herbicide ude in agriculture. These aspects raise questions regarding potential environmental impact of herbicides to biota.

Despite their long use since the 1950s, insufficient data are available regarding risks to sensitive species, while derivatives from new formulation (as 2-ethylhexyl ester, butyl esters, isooctyl ester and other ester or amide forms) (Islam et al., 2018) are even less studied in terms of toxicity to biota (Olker et al., 2022; Qurratu and Reehan, 2016). However, data on the persistence of these PhCAs forms in the environment are inconsistent, with some studies suggesting rapid hydrolysis

* Corresponding author at: National Research and Development Institute for Industrial Ecology, INCD ECOIND, 57-73 Drumul Podul Dambovitei, 060652 Bucharest, Romania.

E-mail addresses: diana_puiu@proton.me, diana.puiu@incdecoind.ro (D. Puiu).

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(Patterson, 2004) and others slow hydrolysis (Muszyński et al., 2020; APVMA, 2006). Due to their high solid-water partition (log $P > 5$) and low water solubility, similar to polychlorobiphenyls, the lipophilic derivatives of phenoxyherbicides are predicted to undergo adsorption-desorption processes on particles that act as carriers in running waters (Thit et al., 2022). Besides runoffs and accidental contaminations of surface waters, WWTPs may also represent a source of herbicide contamination, given multiple potential pathways: herbicides manufacturing and handling, use in urban landscapes or within WWTPs for weed control, and accidental discharges into municipal wastewater. Various studies report the presence of phenoxycarboxylic acids (PhCAs) in both influents and effluents of industrial or municipal WWTPs (Nam et al., 2014) (Lopes et al., 2020). Moreover, an important issue is the ability of WWTPs to remove phenoxyherbicides as some studies have reported no removal or even increased herbicide concentration after treatment (Fernández-Fernández et al., 2022) (Köck-Schulmeyer et al., 2013) (Wang et al., 2018) (Knight et al., 2023). Therefore, WWTPs could represent a worldwide risk for aquatic biota through river discharge, alongside diffuse sources such as agricultural runoffs, atmospheric deposition, acid rain, improper herbicide waste disposal, or tile drainage systems (Manoiu and Craciun, 2021; Islam et al., 2018).

Several environmental conventions have emphasized the need to preserve the Danube Delta, a UNESCO World Heritage Site. One of the objectives of the Danube River Protection Convention (1997) is to control the discharge of wastewater and other point and non-point emission sources into the Danube and its hydrographic basin due to high concentrations of nutrients and hazardous substances. The decline in Danube River's quality, especially near the Danube Delta, has direct or indirect consequences on the chemical and ecological status of Black Sea coastal waters (ICPDR, 1997).

In addition to newly developed treatment technologies (flotation, coagulation, chemical oxidation, biological treatment, ultrafiltration, membrane-based processes, advanced oxidation processes) for removing or degrading herbicides from wastewaters, only a few studies have assessed the efficacy of WWTPs in elimminating synthetic auxin (Köck-Schulmeyer et al., 2013; Nam et al., 2014; Ryu et al., 2022; Wang et al., 2018; Knight et al., 2023; García-Galan et al., 2020), compared with the much larger number of studies focusing on pharmaceutics removal (Iancu et al., 2024). Significant differences exist between the processes developed at laboratory scale and those applied at industrial scale.

The environmental impact of WWTPs on surface water ecosystem can be evaluated at large scale by using tools such as Environmental Impact Assessment, Material Flow Assessment, and Life Cycle Assessment (LCA). At smaller scales, the impact is measured damage to aquatic ecosystem components, including toxic effect on organisms, changes in population density at the sediment-water interface, or indirect influences on the phosphorus, nitrogen and carbon cycles. METland is used as a multimetric indicator to assess the impact of the wastewater treatment plant on the receiving river by combining effects of toxicity, acidification, and eutrophication (Peñacoba-Antona et al., 2021). However, none of these methods incorporates WWTPs efficiency in micropollutants removal, except the Environmental Relevance of Pesticides from Wastewater Treatment Plants Index (ERPWI). This index qualitatively describes the risk posed by WWTP technological performance to the aquatic ecosystem, based on the relationship among three variables: pollutant concentration, WWTP removal efficiency, and compound toxicity, which induces various effects. These effects are determined by EC50 and LC50 indexes, which are often used in other ecotoxicity analysis indices such as risk quotients (RQ) and hazard quotients (HQ) (Gheorghe et al., 2016; Köck-Schulmeyer et al., 2013), but without direct relevance to WWTP performance.

Accordingly, the aim of this observational study is to (1) assess the occurrence of phenoxycarboxylic herbicides in WWTP effluents according to current European legislation, (2) evaluate the possible impact on surface water biota where effluents containing phenoxycarboxylic herbicide residues are discharged, and (3) determine the impact of WWTPs on phenoxy herbicidesloads in the receiving river. Moreover, the study seeks to address questions raised by results regarding WWTPs efficiency in herbicide removal, operational mechanisms and the analytical method's performance in determining these compounds. The phenoxycarboxylic acids investigated are 2,4-D, MCPA, 2,4-dichlorophenoxybutyric acid (2,4-DB), 4-chlorophenoxyacetic acid (4-CPA) and 4-chloro-2-methylphenoxybutyric acid (MCPB). An additional objective is to identify a potential relationship among these compounds that may transform into homologous derivatives during biological processes.

2. Materials and methods

2.1. Study area, sampling and WWTPs characteristics

For the present study, three types of water samples were collected: influents and effluents from three WWTPs, and surface water at 50 m downstream of the effluent discharge point. The water samples were collected in one campaign, for 4–5 consecutive days in October. It was ensured that all sampling plastic bottles were free of herbicide residues. For confidentiality reasons, the municipal wastewater treatment stations are encoded in this paper as A, B and C. The selected WWTPs are classified as secondary (A) and tertiary WWTPs (B, C), serving more than 50,000 inhabitants. The secondary WWTP consists mainly of mechanical and conventional biological stages with activated biological sludge, while the tertiary WWTPs include nitrification and denitrification units after anaerobic, anoxic and oxic units (A2O). It is important to note that WWTP influents are a mix of sewage waters and city's industrial wastewaters. More detailed data about WWTPs are presented in Table S1. The analyzed surface waters were Danube River and its tributary, the Jiu River.

2.2. Analytical methods

The quantitative analysis of the phenoxycarboxylic acids was performed by gas chromatography using a Thermo Trace GC 1310 system coupled to a TSQ 8000 Evo mass spectrometer. The specific method consists of solid-phase extraction of the analytes on C18 cartridges and in situ derivatization with N-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA). The method validation, shown in **Table S2,** was presented in previous papers (Puiu et al., 2019) (Puiu et al., 2020). The method quantifies only the acidic form of the phenoxycarboxylic acids, considering that after 1 h of sample acidification to *p*H 2, all easily hydrolisable forms such as amino, salt and esters derivatives will hydrolyze to the acidic form. The analysis of the water samples was performed on 100 mL influent and 200 mL effluent, respectively surface water. The main steps consisted of homogenization, acidification, and extraction without prior filtration. The results were corrected by recovery. Matrix influence was tested on filtered influent water samples contaminated with 1 μg/L 2,4-DB, 2,4-D, MCPA and MCPB. The results (**Table S2**) correlate with the tendency of compounds to adsorb to organic matter (Paszko et al., 2016).

2.3. Estimation of removal efficiency

The herbicides' apparent removal efficiency of WWTPs was estimated based on the mean concentration (ng/L) difference between effluents ($c_{i,eff}$) and influents ($c_{i,inf}$) without taking into account the inlet and outlet flow rates, according to Eq. (1).

$$\textit{Apparent WWTP removal efficiency}\ (\%) = \frac{C_{i,inf} - C_{i,eff}}{C_{i,inf}}\ x\ 100 \qquad (1)$$

2.4. The WWTP efficiency relevance assessment method

The impact of WWTPs was assessed by using the ecotoxic index ERPWI, developed by Kock-Schulmeyer et al. (Köck-Schulmeyer et al., 2013). The index was calculated by Eqs. (2) and (3) for the most sensitive aquatic organisms to the selected phenoxyherbicides, according to the ECOTOX database (Olker et al., 2022):

$$TUp = \frac{c_{i,eff}}{EC50\ or\ LC50} \tag{2}$$

$$ERPWI_i = TUp \times S_{rem} \times 1000 \tag{3}$$

EC50 or LC50 – the compound concentration at which 50% of the sensitive population is affected by specific (EC) or lethal effect (L) in acute or chronic exposure,

TUp - the ratio between pollutant concentration in *effluent* ($c_{i,eff}$) and the toxicity endpoint EC50 and LC50 (mg/L).

S_{rem} - the score based on the WWTP efficiency (%) in removing the monitored compound (**Table S3**). $ERPWI_i$ – the environmental index assessing the toxicological impact of compounds based on WWTP efficiency, classified as negligible, low, moderate, high or very high (Köck-Schulmeyer et al., 2013).

2.5. Herbicides mass load and grey water footprint

The contribution (%) of WWTPs discharges to surface water herbicide load was calculated by Eqs. (4) and (5).

$$mass\ load\ (g/day) = \frac{c_i\ x\ fr}{10^6} \tag{4}$$

$$Contribution\ (\%) = \frac{mass\ load_{WWTP}}{mass\ load_{river}}\ x\ 100 \tag{5}$$

Mass load – herbicide loading (g/day) in WWTP effluents or surface waters,

c_i – determined concentration of PhCAs in wastewater effluents and surface waters (ng/L),

fr – daily flow rate (m^3/day) of rivers according to the National Institute of Hydrology and Water Management (INHGA, 2019), the daily discharge flow rate of effluents into the selected rivers, or the average daily inflow to WWTPs. Data areshown in **Table S4.**

Grey water footprint (WF) assessment was calculated based on (Hoekstra et al., 2011) using Eq. (6). Results are expressed as m^3/day. The level of pollution is assessed based on the remained assimilation capacity of the rivers (Eq. (7)). River flow data were collected from the relevant authorities, to the sampling period.

$$WF_i = \frac{L_i}{C_{max} - C_{nat}} \tag{6}$$

WF_i - grey water footprint index (m^3/day),

L_i - individual herbicide mass load (g/day) in WWTPs effluents,

C_{max} - maximum admissible concentration of pollutants in surface waters (g/m^3),

C_{nat} - natural background concentration in surface waters (g/m^3)

$$RAC = \frac{WF_i}{AAC}\ x\ 100 \tag{7}$$

RAC - remaining assimilation capacity of the freshwater body (%).

AAC - available assimilation capacity of the freshwater body as river flow (m^3/day).

2.6. Statistical analysis

The significance of the results between influent and effluent sets for each compound and each WWTP was determined using Student's *t*-test, according to expected conditions of normality and variance significance (Shapiro-Wilks test and Fisher-test). To assess the influence of each data set for each compound in influent and effluent, unifactorial analysis (ANOVA and Kruskal-Wallis) were performed. For the case of interest, the data were logarithmically transformed. Statistical analysis was performed using R programming.

3. Results and discussion

3.1. Characterization of herbicides levels and occurrence in selected WWTPs wastewaters

The level of herbicides in wastewater samples was determined for three municipal WWTPs that are closer to agricultural areas. Table 1 presents the herbicide concentrations determined in wastewater samples collected during 4–5 days in a one-time sampling campaign. The sampling days number was selected to avoid the data fluctuation of single-point time analysis. However, campaign analysis represents a preliminary research study about the presence of PhCAs in WWTPs water samples.

The studied chlorophenoxycarboxylic acids were frequently detected. In most cases, their detection frequency exceeded 50% in influents and effluents of municipal WWTPs. All the compounds were detected at ng/L levels, where the highest concentration was 223.7 ng/L MCPA in influents, and 246.7 ng/L 2,4-D in effluents.

The quantified data for effluents are similar to other findings for other Romanian WWTPs in the case of 2,4-D (5–189 ng/L), and higher for MCPA, MCPB and 2,4-DB (>1–6 ng/L) (Norman, 2020). Also, the 2,4-D and MCPA mean values are similar to other worldwide WWTPs effluents (7.64–46.3 ng/L) (Fernández-Fernández et al., 2022) (Köck-Schulmeyer et al., 2013) (Loos et al., 2013).

The results cover only a one-time sampling campaign in autumn and not a seasonal variation. Due to intensive application of phenoxyherbicides in spring, it may be possible to quantify higher amounts of PhCAs in the environment samples during the summer time, as other studies observed (Köck-Schulmeyer et al., 2013), and less in cold seasons.

Therefore, the presence of 2,4-D, MCPA, and MCPB in wastewater samples may be linked to agricultural sources, due to their use as active ingredients in herbicide products (**Table S5**), considering that PhCAs half-life time in water could be over 40 days from the spring application (Kuster et al., 2008) (Nam et al., 2014). On the other hand, the presence of 2,4-DB and 4-CPA needs greater attention because their use is limited by European and national legislation (Pesticides EU database, 2023).

However, by choosing the sampling period in October, it was expected to obtain a glimpse of the herbicides presence in WWTPs waters and related surface waters outside the agricultural time window. The results may show water contamination thorough other sources such as a) various herbicide waste discharges, b) herbicide production industry or c) in situ transformations from other related compounds or by the slow kinetic rate of other PhCAs biodegradation.

For example, all three WWTPs receive municipal wastewater from combined sewer systems, mixed with industrial wastewaters from small- and medium-sized enterprises. These companies can use PhCAs as herbicides to control various vegetation or as auxins to accelerate the hormonal growth of edible plant ripening, such as tomato (Li et al., 2017) or apple (Kondo et al., 2009) (Karas et al., 2016). Also, some operators may treat the sewer pipe lines with herbicides to inhibit tree roots infiltration and vegetation growth (Widhiastuti et al., 2023). However, the usually applied concentrations are higher than 1 mg/L, and are not expected to be related to the ng/L levels detected in influents. The results could not be assigned to the herbicide production industry in Romania because all known herbicide products based on phenoxy derivatives are imported.

The estimated herbicide mass load in the inflow is presented in **fig. S1**, reported per number of inhabitants. The similar profile of herbicide ratio for WWTP B and C could be correlated with the cities agricultural activities. The contamination with MCPA is mostly visible for WWTP A

Table 1
Results of herbicides concentration (ng/L) as mean, median, minimum, maximum and frequency for all selected WWTPs.

	influent					effluent				
	mean[1]	median	min[2]	max	frequency	mean[1]	median	min[2]	max	frequency
2,4-D	32.1 ± 51.7	11.1	<3	164.2	53.8%	61.2 ± 77.8	34.6	4.66	246.7	100%
4-CPA	24.0 ± 30.3	19.4	<3	114.7	61.5%	36.0 ± 43.5	27.0	<3	142.4	76.9%
MCPA	76.9 ± 70.6	53.1	8	223.7	100%	33.1 ± 31.4	30.0	<3	129.7	84.6%
2,4-DB	18.3 ± 23.9	10.0	<3	73.7	46.2%	23.1 ± 32.3	9.0	<3	100.0	76.9%
MCPB	10.5 ± 9.3	7.4	<3	24.1	23.1%	8.7 ± 12.5	< 3.0	<3	47.0	30.8%

[1] the values below the method quantification limits (LOQ) were included in the average calculation.
[2] the value represents the LOQ for the GC method.

and B. The obtained data is comparable with other studies. For example, the input mass load for Seoul City was identified to be 15.3 mg 2,4-D/day x 1000 inhabitants (Nam et al., 2014) compared to 13.4 mg 2,4-D/day x 1000 inhabitants determined for WWTP B.

3.2. WWTPs apparent performance in herbicide removal

Based on statistical analysis for each WWTP results, the compounds concentrations do not differ significantly between influents and effluents, in terms of mean and variance (*t*-test), except for MCPA in WWTP A ($p < 0.05$). At a first glance, the selected WWTPs do not favour the removal of the phenoxycarboxylic compounds at this concentration level. However, herbicide means concentrations slightly increase in effluents compared to influents, as it is expressed in Fig. 1, calculated with Eq. (7).

A closer look at WWTPs results (Fig. 1, **Table S6**) reveals that the applied treatment has three effects: removal, no effect and accumulation of the herbicide concentrations in effluents. Only MCPA was removed in all treatment stations (29.9–77.5%), while 2,4-D and 4-CPA concentrationd were diminished in WWTP A. Unexpectedly, the other auxins results show an increase of concentrations in effluents, where the highest percentage difference is 253.9% for 4-CPA. The removal trend is consistent with other published data for acidic herbicides (Nam et al., 2014) (Köck-Schulmeyer et al., 2013) (Ryu et al., 2022) (Hill et al., 1985), while other studies show increase of MCPA and other xenobiotic as pesticides, herbicides and pharmaceutics with similar molecular structure (clofibric acid, enrofloxacin, metoprolol etc.) (Fernández-Fernández et al., 2022) (Köck-Schulmeyer et al., 2013) (Wang et al., 2018) (Knight et al., 2023).

The results for selected WWTPs at this level of technology are similar with those of biological treatment with algae (García-Galan et al., 2020), such as the increase of 2,4-D content and the removal of MCPA. On the other hand, by spliting the technology into simple or cumulative operational physical-chemical treatment steps (filtration, chlorination, coagulation, etc.), it does not results an increase of 2,4-D in effluent (**Table S7**) (Lopes et al., 2020) (Köck-Schulmeyer et al., 2013) (Feng et al., 2025). At request, the data for each station will be provided.

3.3. Discussion on PhCAs accumulation in WWTPs effluents

The accumulation of PhCAs in WWTPs does not support the hypothesis that herbicides, along with other organic compounds, are degraded through treatment processes. Therefore, the most suitable explanations for the increase in outflows of acidic herbicide amount, from the perspective of PhCAs derivatives (esters, amides, salts, etc.), in correlation with analytical techniques specificity, *in situ* transformations between PhCAs, and the design of WWTP biological units.

3.3.1. Analytical technique design versus hydraulic retention time

It was noticed that it may be possible to quantify a higher amount of PhCAs if the water samples are acidified below *p*H 2, when the compounds are in their protonated form (pKa: 2.7–4.95) (Kim et al., 2025).

This statement takes into account other studies that use solid-phase extraction method and GC–MS analytical determination. The main parameters identified as defining the analytical technique design are:

a) Extraction *p*H. The variation of *p*H to 2, 2,8 and 4 may give different extraction recoveries due to solubility constant. These are the *p*H values at which the protonated acids were extracted in other research studies by adsorption onto lipophilic cartridges (Wang et al., 2018; García-Galan et al., 2020; Hill et al., 1985; Ryu et al., 2022; Knight et al., 2023; Köck-Schulmeyer et al., 2013) (Wang et al., 2020) (Yang et al., 2017). Even though the dissociation yield does not directly influence herbicide degradation in WWTPs, a more frequent correlationwas found between PhCAs accumulation at *p*H 2 than at *p*H 4 (Table S8). For example, at *p*H 2 an increase in effluents of 242% for 2,4-D and 324% for clofibric acid was reported, consistent with the data obtained in this study.
b) Presence of easily hydrolysable phenoxyherbicides species, such as salts, amides and esters (Garnayak et al., 2024), which can be transformed into acidic forms. This process occurs by acid catalysis during samples pretreatment at acidic *p*H for analytical

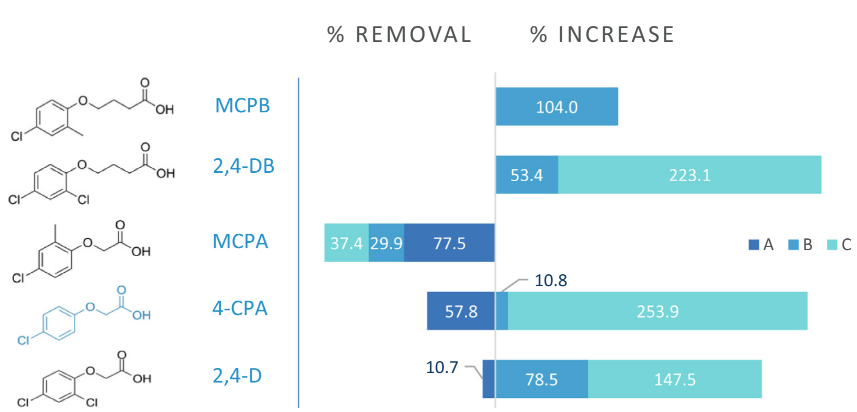

Fig. 1. Apparent removal efficiency of herbicides (by mean concentration of 4–5 days, calculated by Eq. (1)) for each targeted WWTP.

determination. The most common 2,4-D derivatives expected in WWTPs due to high agricultural use are 2,4-D butyl ester (2,4-D BE), 2,4-D isooctyl ester (2,4-D IOE) and 2,4-D ethylhexyl ester (2,4-D EHE) (Islam et al., 2018). However, comparing hydrolysis half-times shows significant variation: 0.6 h (2,4-D BE), 33 h (2,4-D IOE), and 35 days (2,4-D EHE) (Muszyński et al., 2020).No studies were identified reporting the presence of these esters in wastewater samples.

Considering that *p*H variation in the analytical method doesn't imply different recovery for PhCAs from influent and effluent, it may be concluded that increased concentration in effluents may be determined by the analytical extraction method correlated with the biotransformation half-time of PhCAs during biological treatment and the hydraulic retention time of WWTP biological stage (HRT_{bio}). In other words, the decreases in extraction *p*H correlated with hydrolysis time and HRT_{bio} provides insight into the type and quantity of phenoxy auxin derivatives. Moreover, questions may arise regarding the partitioning of PhCAs derivatives between water, sludge and air bubbles due to hydrophobic characteristics (Burzio et al., 2024).

3.3.2. Biological unit design

3.3.2.1. Increased concentration by transformation. It is expected that the main transformation of PhCAs will take place in the biological reactors (Verma et al., 2014), because removal through primary filtration or chemical oxidation does not significantly affect micropollutants (Lopes et al., 2020) (Nam et al., 2014) (Hill et al., 1985). In this study, the biological reactors of the WWTPs follow the configuration of the 3-stage modified Bardenpho system: a sequence of anaerobic, anoxic and aerobic treatment units (Chen et al., 2020), as Shown in Fig. 2.

PhCAs may undergo transformations between derivatives (salts, amides, esters) or between homologous PhCAs. Moreover, the compounds may be degraded under biological conditions to intermediates, mainly by demethylation, dehalogenation, hydroxylation and oxidation (Singh and Singh, 2014) (Islam et al., 2018) (Muszyński et al., 2020).

For example, 2,4-DB and MCPB, two proherbicides, can lose two carbon atoms by β-oxidation as fatty acids into glyoxylate mechanism, under specific enzymatic conditions, after uptake into biota cells, through successive steps in the aerobic reactor. In anaerobic fermentation, it is more likely that 2,4-D is degraded in a reductive cycle into 4-CPA and phenoxyacetic acid (PAA) by ortho- and para-dehalogenation (Zharikova et al., 2021) (EFSA (European Food Safety Authority), 2016) (Brucha et al., 2021) (Zipper et al., 1999). In the aerobic stage, PhCAs are more likely to be hydroxylated to chlorophenols and hydroxyphenols under specific enzymes (Verma et al., 2014) (APVMA, 2006).

Based on these data, the dynamic of PhCAs content in wastewater could be reflected by successive transformation into homologous compounds, as shown in Table 2**.** The mass balance for each herbicide was assessed by the difference between the demand amount and generated amount, where the amount represents the sum of the determined PhCAs (mM) for all days, except the first effluent day and the last influent day, due to estimated 24 h HRT. The results (Table 2) show that only for the 2,4-D/4-CPA reaction in WWTP C a possible variation was found, correlating 2,4-D demand with 4-CPA increase. However, the mass balance is not closed, because the high content of 4-CPA suggests additional generation sources. No mass balance was observed for the other cases. Without HRT correction, the results still show an increase in effluents.

The study did not include monitoring of other degradation products such as phenoxyacetic acid or chlorophenols, mainly because their formation, like that of 4-CPA, is linked to other compounds, especially pharmaceuticals such as clofibrate, which are likely to be present in municipal wastewater (Wang et al., 2018). Some authors concluded that metabolites such as 2,4-dichlorophenol or 3,5-dichlorocatechol are

Table 2
Mass balance of main reactions between homologous PhCAs for WWTPs A, B and C.

		A	B	C
$2,4-DB \xrightarrow{k_1} 2,4-D$	2,4-DB:	n.d. / n.q.	↘0.14 mM	↘0.01 mM
	2,4-D:	↘0.11 mM	↘0.22 mM	↘0.04 mM
$MCPB \xrightarrow{k_2} MCPA$	MCPB:	n.d. / n.q.	0.00 mM	n.d. / n.q.
	MCPA:	↘2.27 mM	↘1.05 mM	↘0.37 mM
$2,4-D \xrightarrow{k_3} 4-CPA$	2,4-D:	↘0.11 mM	↘0.22 mM	↘0.04 mM
	4-CPA:	↘0.21 mM	↘0.49 mM	↗0.19 mM

k_{1-3} - kinetic constant rate for diffusion of PhCAs in bacteria cells and reaction.
n.d. / n.q. - not detected / not quantified.

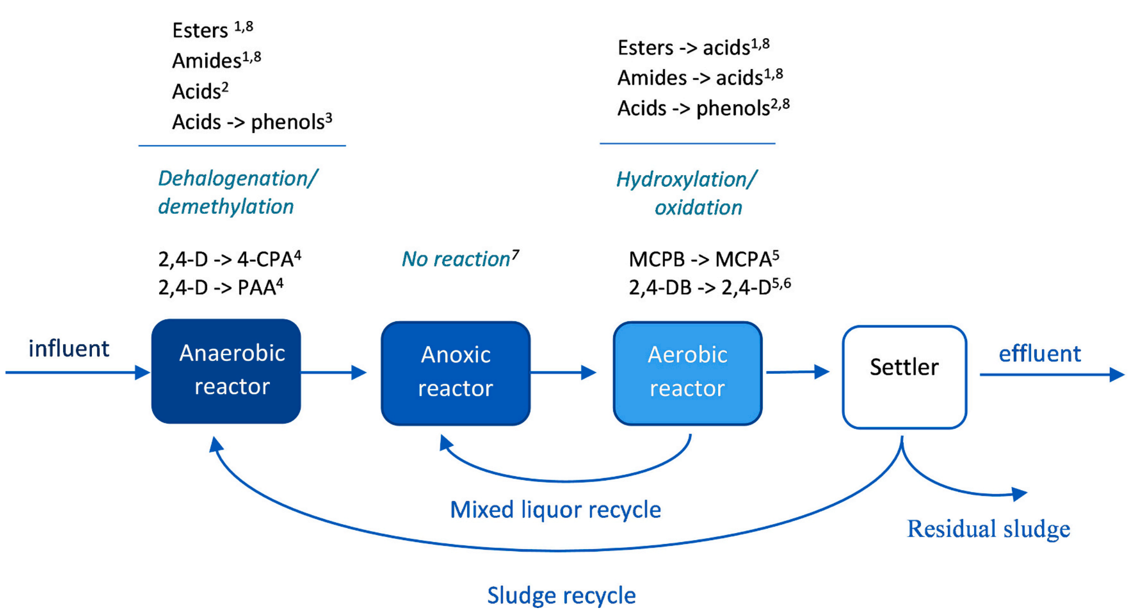

Fig. 2. Schematic representation of typical wastewater and sludge recirculation in biological treatment units: anaerobic, anoxic and aerobic (A2O). [1] (Rajagopalan and Kroutil, 2011), [2] (Verma et al., 2014), [3] (Zipper et al., 1999), [4] (Zharikova et al., 2021), [5] (Paszko et al., 2016), [6] (EFSA (European Food Safety Authority), 2016), [7] (Chen et al., 2020), [8] (APVMA, 2006).

difficult to monitor in aquatic environmental conditions (White et al., 2022).

Another pathway could explain the increase of PhCAs in effluents: the conversion of derivatives to chlorophenols and their transformation back to phenoxy acids. Phenoxy herbicides esters and amides are hydrolyzed into corresponding acids in aerobic reactor, and to a lesser extent in anaerobic fermentation, under enzymatic catalyst of lipases and proteases (Rajagopalan and Kroutil, 2011). The metabolization of phenoxy herbicides acids as carbon and energy sources for bacterial growth generates conjugated esters of PhCAs such as glutamates, aspartates, acetyl herbicides-coenzyme A and acetyl herbicide-phosphate which degrade to chlorophenols andhydroxy derivatives. Under certain conditions, these metabolites convert back to free phenoxy acids (Eyer et al., 2016) (Li et al., 2003) (Whicher et al., 2018).

3.3.2.2. Increased concentration by recirculation. It is expected that the transport time of acids through the WWTP is equal to the hydraulic retention time due their high solubility in water, followed by removal in effluents. However, the presence of PhCAs as lipophilic derivatives may require more time than one HRT_{bio} cycle (Fig. 3) due to the sorption onto organic matter (Burzio et al., 2024). This means that the first HRT_{bio} effluent will contain only the acidic derivatives, while the second and subsequently HRT_{bio} cycles will contain ester derivatives from the first HRT_{bio} cycle, after desorption. Therefore, the amount of phenoxyherbicides in wastewater will follow an unpredictable variation.

The analysis of herbicide concentrations in effluents (Fig. 4) over several days, revealed a decrease that appears to fit second-order kinetic removal rates, with good correlation (>0.90 for WWTP B and C, >0.85 for WWTP A). This indicates the presence of multiple reversible transformations strongly influenced by the quantity of reactants. However, the PhCAs kinetic removal rate is slow, up to 2.0×10^{-7} M^{-1} s^{-1} for 2,4-DB in WWTP B.

To assess whether the mean increase of PhCAs in effluents is based on ester adsorption and sludge recirculation, the daily concentration of PhCAs in effluents was simulated by using Eqs. (8)–(9). The results, displayed in Fig. 4**.,** show 2,4-D decay over time for each WWTPs as a function of sludge recirculation and daily influent load. The calculated concentration represents an estimated fraction of each analyte, assuming no degradation in biological units, with 30% returned through sludge recirculation and 70% ± 20% discharged in effluent every 24 h. The equations do not include the analyte mass transfer through mixed liquor suspended solids recycle.

$$C_{se,j} = 70\% \ x \ C_{si,j} \tag{8}$$

$$C_{si,j} = C_{ri,j} + 30\% x \ C_{si,j-1} \tag{9}$$

C – analyte concentration for influent (i) or effluent (e), for simulated conditions (s) or real determination (r).

i – day number, $i > 2$.

The compound 2,4-D was selected because of its persistence compared to other PhCAs in the biological unit accordingly to obtained data. The predicted results show good correlation with the experimental data for WWTP B. For WWTP A and C, the similarities diminish on day 3 or 4, when 2,4-D concentration increases, possibly due to biodegradation of 2,4-DB to 2,4-D or because the 24 h sludge recycle is not relevant in removing 2,4-D·As a proof, later analysis revealed the presence of 2,4-D in dried residual activated sludge at 52.4 μg/kg total solids. Unlike 2,4-D and 4-CPA, MCPA tendency does not fit the prediction, possibly due to its lower resistance to biodegradation.

3.4. Toxicological relevance of WWTP efficiency on herbicide removal in aquatic ecosystems

3.4.1. ERPWi index

The ecotoxicological impact of WWTP wastewater discharges on surface water biota was assessed based on removal efficiency of PhCAs and compound toxicity to sensitive organisms. For this evaluation, the environmental index ERPWi was applied, which includes toxicological data based on hazard and exposure, used in European Environmental Agency reports (Carvalho et al., 2015). The ERPWi index was calculated (Eq. (3)) for the most sensitive aquatic species to phenoxy herbicides according to the ECOTOX database, from each trophic class (Olker et al., 2022), and for aquatic species usually found in the Danube and Jiu Rivers, including endangered or locally dissapeared species.

These species were classified as phytoplankton (diatomee, algae), macrophytes, invertebrates and vertebrates. The index was determined for median and maximum concentration of the analyzed herbicides from all WWTPs. The results are presented in **Table S9**, while the lowest ERPWi index for each trophic class is shown in Fig. 5.

It was revealed that the highest potential impact can be exerted by 2,4-D and MCPA, as acids or salts, on phytoplankton. Under acute exposure (2 days), to acidic form strongly affects the diatom Gomphonema, while under chronic exposure (14 days), the sodium salt of 2,4-D induces toxic effects in green algae such as *Chlorella pyrenoidosa*, *Dictyosphaerium pulchellum* and *Chlorococcum* species.

At higher trophic levels, toxicity tends to decrease from very high toxicity for phytoplankton and macrophytes (*Myriophyllum spicatum* or *Lemna Gibba*) to moderate toxicity for invertebrates and fish. Although complex organisms such as invertebrates and vertebrates show higher resistance. Species such as *Oncorhynchus mykiss* and *Oreochromis niloticus* are at high risk of mortality when exposed to the maximum determined concentrations of 2,4-D, respectively MCPA.

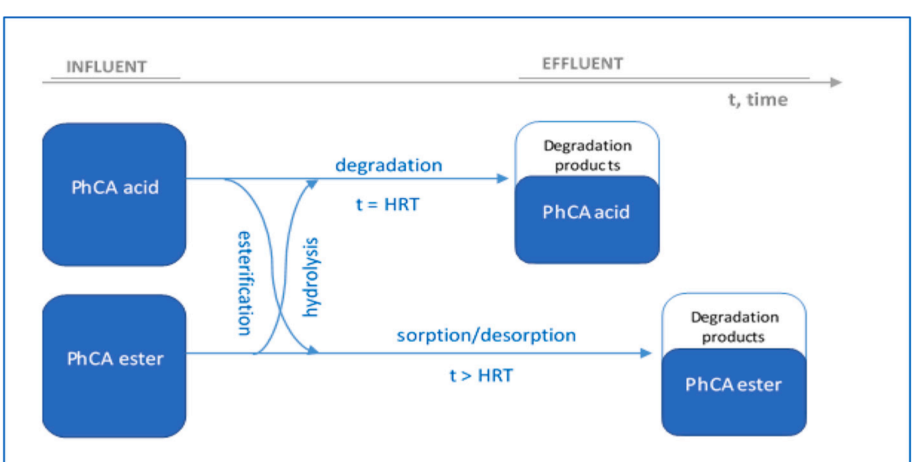

Fig. 3. The proposed mechanism of PhCA acids and esters in WWTP.

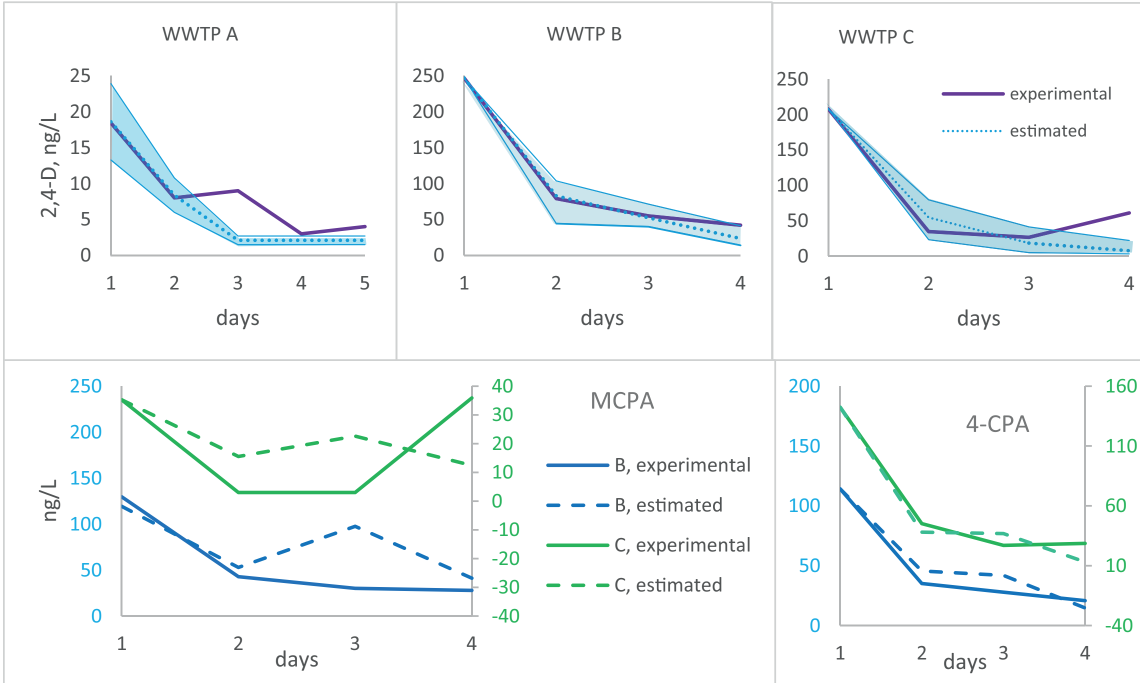

Fig. 4. Experimentally determined decay of 2,4-D over time versus simulated decay of 2,4-D for A2O treatment in WWTPs A, B and C, and experimental decay of MCPA AND 4-CPA versus prediction for WWTP B and C.

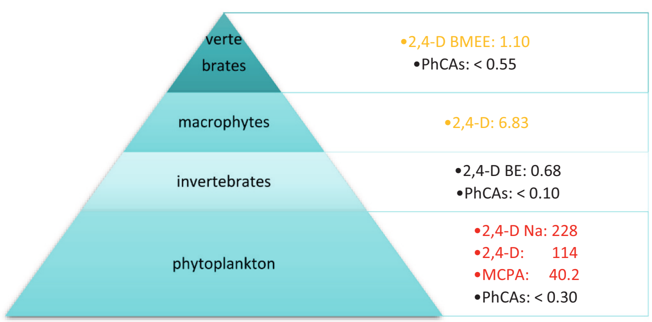

Fig. 5. The ERPWi values associated with the main aquatic biota classes, calculated from mean of PhCAs concentration from WWTPs A, B and C, where red, orange and black indicate very high, high and medium impact classes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The toxicity data for ester derivatives of PhCAs do not cover all trophic classes. However, among 2,4-D esters such as 2-ethylhexyl ester (2EHE), isooctyl ester (ISO), butyl ester (BE) or butoxymethylethyl ester (BMEE), only 2,4-D BMEE induces high toxicity to fish *Oncorhynchus clarkii* under chronic exposure. Nevertheless, phenoxy derivatives hydrolyze in water to acidic forms which are highly toxic to phytoplankton and some macrophytes, and toxic to several vertebrates. In comparison, MCPB and 2,4-DB show lower toxicity for the same species. Moreover, toxic impacts on phytoplankton may indirectly affect species at higher trophic levels.

The relevance of these sensitive aquatic species to the Danube and Jiu River ecosystems allows the classification of biota as:

- endangered species to phenoxy herbicides (**Table S9**), not identified in the Danube water body or tributaries, such as *Daphnia Magna*, a wide-spread crustacean and ecotoxicity indicator, found only at the sediment surfaces. However, multiple intoxication sources exist for*Daphnia* species beyond moderate effect of 2,4-D and MCPA at concentrations above 100 μg/L (Stoica et al., 2014) (Liska et al., 2021).
- endangered species to phenoxy herbicides determined in this study (**Table S9**), abundant in the Danube and tributaries, such as *Gomphonema* species (*Gomphonema minutum, Gomphonema parvulum*) and *Achnanthidium minutissimum* (an aquatic invasive plant) (Buczko et al., 2022). Their presence and abundance can serve as eco-indicators for 2,4-D and MCPA toxicity.
- species with unknown risk according to ECOTOX, including benthic species with relative abundance >1% in the studied rivers: Bacillariophyta (*Navicula tripunctata, Navicula antonii, Cocconeis placentula, Rossithidium anastasiae, Melosira varians, Navicula gracilis, Diatoma elongatum, Synedra acus*), Chlorophyta (*Pediastrum boryanum*), *and* Mollusca macroinvertebrates(*Dreissena polymorpha, Lithoglyphus naticoides)* (Stoica et al., 2014) (Olker et al., 2022) (Liska et al., 2021).

The ERPWI index is not an absolute ecotoxicity tool because EC50 and LC50 values do not cover long-term exposure (minimum 14 days) or different commercialized auxin esters. Also, the ERPWi does not account for synergistic effects of herbicides mixture.

Although, the ERPWi index was calculated based on PhCAs concentrations in WWTP effluents, dillution in receiving rivers may significantly reduce the potential impact. Therefore, ERPWi should be considered a warning indicator of WWTP risk capacity.

3.5. Herbicides loads WWTPs contribution to discharging rivers

In this study, the phenoxycarboxylic acids were detected in the Jiu River and Danube River with mean concentrations below 100 ng/L (**Table S10**), the limit established by Romanian and European directives (Order 161/2006 and UE Directive 2184/2020) for surface water and drinking water, and below the predicted no-effect concentration (PNEC) of 20 ng/L (Olker et al., 2022). However, an exception was observed for 2,4-D in the Jiu River, with a mean of 128.5 ng/L and a maximum of 309 ng/L. The mean values of 2,4-D and MCPA are comparable with other data reported for the Danube River and its tributaries (Loos et al., 2010)

(Norman, 2020) (Ng et al., 2023), as well as for other surface waters in Europe and Canada (**Table S11**) (Feng et al., 2024) (Feng et al., 2025) (Székács et al., 2015) (Casado et al., 2018) (Woudneh et al., 2007; Gamhewage et al., 2019). Still, the maximum values were below the historical maximum 2,4-D concentration determined in the Danube in 2010 (Loos et al., 2010). Phenoxy herbicides are rarely monitored in environmental samples in Eastern European countries (Norman, 2020), mostly because 2,4-D, MCPA, 4-CPA, MCPB and 2,4-DB are not listed as dangerous compounds in the EU Watch List (EU 2022/1307) or as hazardous under Directive 2008/105/EC for surface water environmental quality standards.

To assess the contribution of WWTPs herbicide loads to receiving rivers, the grey water footprint index was analyzed, considering that the amount of herbicides in surface water is already assimilated by the water basin and included in Eq. (6) as C_{nat}. C_{max} was set at 100 ng/L, the maximum admissible concentration of herbicides in drinking water in Romania. The determined herbicide mass load (Eq. (4)) from WWTP effluents ranged from 0.17 to 8.6 g/day. However, based on the grey water footprint index, effluents dilution strongly influences the available assimilation capacity of the selected rivers. The remaining assimilation capacity for phenoxy herbicides (Eq. (7)) is at least 99.3% for Jiu River, and above 99.9% for the Danube (**Fig. 6**).

Overall, the WWTP contribution of phenoxy herbicides to both rivers is low, with the sum of herbicides (mean) below 0.2% for the Danube and 0.6% for the Jiu. Their presence in surface water may result from agricultural runoff, WWTP discharges, or unknown point sources (Islam et al., 2018).

Even if the footprint index is not significant, higher contamination was observed in the Jiu River compared to the Danube, with the highest PhCA contribution being 0.343% for MCPA. However, when extrapolating to the entire water body, the herbicide mass load in the Danube is significantly higher than in the Jiu River: 12.4 kg/day 2,4-D mean or 7.3 kg/day MCPA mean at the sampling point before the Danube Delta, compared to 0.25 kg/day 2,4-D mean and 0.12 kg/day MCPA mean in the Jiu River. The values are comparable with findings for perfluoroalkyl substances (PFAS) din a similar Danube segment (Beggs et al., 2023).

As shown, WWTPs are most likely not the main sources of river contamination with herbicides, consistent with other studies (Ryu et al., 2022).

4. Conclusions

This preliminary study raises a warning about the occurrence of phenoxy herbicides in wastewater effluents and receiving rivers at levels of hundreds of ng/L.

As expected, the tertiary WWTPs are not designed to efficiently remove micropollutants such as phenoxy herbicides. On the contrary, increased PhCAs concentrations were observed in effluents. However, the results are limited by the temporal and spatial representativeness of the sampling campaign. Therefore, further studies are necessary to assess WWTP contamination of WWTPs with phenoxy herbicides over longer periods, considering seasonal variation.

To evaluaet the robustness of the results, the data were statistically interpreted, considering the limitations of analytical methods and the presence or absence of reaction mechanism in WWTP processes. The main conclusions are:

a. The daily variation of herbicides from influents compared to effluents is not statistically significant.
b. The influence of extraction *p*H associated with the hydrolysis rate of herbicide derivativeswas theoretically explored. The conclusion is that biological treatment may convert the low hydrolysable ones, which could explain the high concentrations in effluents.
c. Assuming no reaction mechanism occurs in herbicide degradation, the results were estimated based on the recirculation design of the secondary treatment process (A2O). The proposed equation, dependent on influent concentration and hydraulic retention time, shows similar results for 2,4-D and 4-CPA in effluents.

Fig. 6. Assimilation capacity of rivers based on grey water footprint assessment of Danube and Jiu River loads with herbicides from WWTP discharges.

This study clearly demonstrates the need of an additional treatment step to improve WWTP efficiency in removing micropollutants. Moreover, as indicated by the ERPWi risk assessment index, phenoxy herbicides may have a very high toxic impact on diatom species and some macrophytes in aquatic ecosystems. Unfortunately, the relevance of derivative forms cannot be assessed similarly to acidic herbicides due to the lack of toxicological data.

However, the impact of the studied WWTPs to surface water is considered low according to grey water footprint index, because the assimilation capacity of the rivers exceeds 99%.

Following studies should assess the impact of WWTP processes, hydraulic retention time and wastewater flux on the removal of non-targeted micropollutants and their derivative forms.

CRediT authorship contribution statement

Diana Puiu: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Corina Bradu:** Writing – review & editing, Visualization, Validation, Methodology, Conceptualization. **Costel Bumbac:** Writing – review & editing, Validation, Conceptualization. **Lucian Constantin:** Writing – review & editing, Validation, Resources, Funding acquisition. **Lidia Kim:** Writing – review & editing, Validation. **Olga Tiron:** Writing – review & editing, Validation. **Carmen Postolache:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, 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.jconhyd.2026.104903.

Data availability

Data will be made available on request.

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