ACUTE AND CHRONIC ECOTOXICITY OF A PHARMACEUTICAL EFFLUENT ON *DAPHNIA MAGNA* IN MOROCCO

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Abstract. The goal of this study is the ecotoxicity evaluation of an effluent from a pharmaceutical manufacturer in Morocco on freshwater cladoceran species *Daphnia magna.* The water flea was exposed to pharmaceutical effluent of different concentrations for acute and chronic assessment. Chronic bioassays were performed using survival, reproduction, and body length endpoints after 21 days of exposure. The physicochemical pollution was also evaluated using conventional parameters. The results of the chemical analysis indicated that pharmaceutical effluent was chiefly polluted with organic, nitrogenous, and sulfate compounds as expressed by the value of COD (280 mg/L), TN (5.38 mg/L) as well as sulfate (110 mg/L), respectively. However, all measured parameters are in compliance with general permissible limits for discharges into surface waters recorded by Moroccan legislation. For toxicity results, the effective concentration immobilizing 50% of *Daphnia magna* after 24 h was 22.65% v/v ± 0.61. The results of the chronic biotest demonstrate that the numbers of juveniles, body length, and brood size were increased especially at two higher concentrations compared to control. This stimulation could be related to the presence of endocrine distributors linked to the pharmaceutical production process as it alters directly the fecundity of water flea.
Keywords: *pharmaceutical effluent, Daphnia magna, acute toxicity, chronic toxicity, physicochemical characterization*

Introduction

Since the introduction of a new industrial acceleration policy in 2014, the pharmaceutical industry in Morocco is quickly growing. The sector constitutes the second industrial activity, which makes the country the second-largest African pharmaceutical manufacturing industry after South Africa. In the current day, Morocco has nearly 40 pharmaceutical companies with a capacity production of 350 million units per 8-h shift (Ladid and Houssni, 2022). This wide capacity raises concerns about the large amount of wastewater produced during the manufacturing process of pharmaceutical compounds (PhACs).

Generally, it is recognized that effluents from municipal wastewater treatment plants and hospitals are the major sources of the emergence of PhACs in aquatic receiving environments (Chiffre et al., 2016). However, recent evidence reveals that wastewaters from pharmaceutical factories-discharged are relevant sources of pharmaceutical contamination (Cardoso al., 2014). Their organic nature is generally complex and

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contains a wide range of raw chemicals, by-products, solvents, and other ionic compounds (Pérez et al., 2017). The conventional process, which represents 70% of Moroccan treatment process, is insufficient to complete the removal of active ingredient pharmaceuticals (Imwenea et al., 2022). As a result, this situation implies strict control of discharged effluent quality.

Several studies have confirmed the presence of PhACs in unaltered form or as a metabolite in sewages, surface and ground waters as well as potable water (Liu et al., 2022; Wang et al., 2021; Ma et al., 2022; Kodom et al., 2021). Mastrángelo et al. (2022) detected various therapeutic classes including analgesic, antibiotic, antihypertensive, β-blocker, diuretic, and psychiatric drugs in lowland urban rivers in Argentine at concentrations between 99 ng/L and 9622 ng/L. However, the data concerning the potential ubiquity of PhACs in aquatic matrices in Morocco are limited. Chafi et al. (2022), reported the first study in the river Bouregreg (Rabat, Morocco). In their publication, a high detection frequency (80%) was reported for anti-inflammatory/analgesic drugs, followed by antibiotics and anti-epileptics (64%), lipid regulators (56%), and β-blockers (12%).

The occurrence of PhACs in freshwater ecosystems raises increasing concern about their toxic effects on aquatic wildlife (Milić et al., 2013; Machado and Soares, 2019). Several studies demonstrate the acute and chronic toxic effects on bacteria, microalgae, microcrustaceans, and fishes exposed to trace levels of PhACs (Xie et al., 2017; Bielen et al., 2017). In addition, long-term exposure to environmental concentrations of PhACs can affect the biological process of aquatic animals such as behavioral changes (Hong et al., 2021; Grabicova et al., 2017), oxidative stress (Duan et al., 2022), reproductive cycle (Fischer et al., 2021; Branco et al., 2021) as well as hormonal changes (Shreenidhi et al., 2023).

Compared with other invertebrates, the planktonic crustacean *Daphnia magna* (*D. magna*) is commonly used in water quality assessment (Berrebaan et al., 2020; Hashiguchi et al., 2020). This species is recommended by different standards (EPA and OECD) as a model test organism in aquatic ecotoxicology. This widespread use is due to the position in the aquatic food web linking phytoplankton to fish (Miner et al., 2012). Furthermore, the similarity of the mode of action between humans and invertebrates represented by *D. magna* to many drugs could be used as bioindicators of public health effect prediction (Siciliano et al., 2015). Moreover, the population of *D. magna* is characterized by their short time cycle (reaches sexual maturity in approximately 6–8 days), ease of culture, and parthenogenesis reproduction (Miner et al., 2012).

This study is the first in Morocco that aims to evaluate the impact of raw wastewater from a pharmaceutical company on the population of *D. magna.* To achieve this goal, we evaluated the acute toxicity after 24 h of exposure to pharmaceutical effluent. In addition, several reproduction endpoints were investigated after 21 days of exposure. The objective of the present study is also to check the safety of discharging effluents into Moroccan receiving waters.

Materials and methods

Effluent collection and preparation

The raw effluent was collected from a pharmaceutical company in the region of Rabat, Morocco (Western Morocco). The industrial activity of the company is focused

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on the production of generic drugs including antibiotics, cardiovascular, anti-inflammatory non-steroidal, antihistaminic, nervous, and urogenital systems (about 60% of industrial activity).

The sample was collected in 1L of sterile plastic containers. The effluents were stoked at 4°C in darkness.

Physicochemical characterization

The pH, electrical conductivity (EC), and temperature (T) were directly measured on the sampling site by a portable multi-parameter pH/COND/DO meter (WTW Multi 3430, Xylem Group, Weilheim, Germany). In the laboratory, eight chemical parameters were determined for pollution control. The COD was assessed using the potassium dichromate ($K_2Cr_2O_7$) method after digestion. The BOD_5 was assessed from samples diluted via the oximetry method (OxiTop®-i IS6-WTW, Xylem Group, Weilheim, Germany). The Total suspended solid (TSS) was determined using filtration through glass fiber filters. The total nitrogen (TN), ammonium nitrogen (NH_4^+-N), and sulfate were determined by the colorimetric method using continuous flow. The total phosphorus (TP) was assessed by the continuous flow analysis. The concentration of iron (Fe) was measured by the inductive coupled plasma mass spectrometry (ICPMS Agilent 7500a, Agilent Technologies, Santa Clara, USA).

Culturing conditions

Experiments were conducted with strains of *D. magna*. The test organisms were maintained in ISO 6341 medium (containing 11.76 g/L $CaCl_2$ $2H_2O$, 4.93 g/L $MgSO_4$ $7H_2O$, 2.59 g/L $NaHCO_3$, 0.23 g/L KCL) at a temperature of 20 ± 2°C and under ambient light (NM ISO 6341, 2014). The culture medium has a pH of 7.8 ± 0.2, a total hardness of 250 ± 25 mg/L, and a conductivity of 10 µS/cm. The culture medium was renewed three times a week and daphnids were fed twice per day with a mixture of three algal species (5×10^6 *Pseudokirchneriella subcapitata*, 2.5×10^6 *Scenedesmus subspicatus*, 2.5×10^6 *Chlorella vulgaris*/Daphnia/day) and a synthetic food (TetraMin).

Acute toxicity test of D. magna

The acute ecotoxicity test with *D. magna* was performed using immobilization as an endpoint according to Moroccan standards (NM ISO 6341, 2014). Juveniles less than 24 h old from parthenogenesis females were exposed to ranges of concentrations (8.5, 12.5, 18.75, 28.12, 42.18, 63.27, and 94.97% v/v).

Five neonates of *D. magna* were placed in 15 mL glass test tubes. The glass tubes contain 10 mL ISO medium and the concentrations tested. The test was performed with four replicates for each concentration and control. The test tubes were incubated under static conditions and complete darkness at 20 ± 2°C. The neonates were not fed during the biotest.

After 24 h of exposure, the immobility of *D. magna* was observed. Neonates still moved their antennae but are unable to swim within 15 s after a gentle shaking was considered immobile.

Chronic toxicity test with D. magna

The chronic toxicity was performed in accordance with Moroccan standards (NM ISO 10706, 2014). In the chronic toxicity test, daphnids less than 24 h old were exposed

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for 21 days to measured concentrations of pharmaceutical effluents (0.067, 1, 1.25, 1.88, 2.82, 4.24, and 6.63% v/v). Neonates were individually placed in small crystallizers of 50 mL containing 40 mL of the culture medium and effluent at tested concentrations. The experiments were performed in ten replicates for each concentration and control. All crystallizers were maintained at 20 ± 1°C and a photoperiod of 16/8 h.

D. magna were fed daily with a mixture of three algal species 5×10^6 *Pseudokirchneriella subcapitata*, 2.5×10^6 *Scenedesmus subspicatus*, and 2.5×10^6 *Chlorella vulgaris*/Daphnia/day). All test beakers were checked daily for parental mortality and offspring production of neonates. The reproductive endpoints assessed were longevity, body length, days to first brood, total number of neonates per female, and brood size.

Statistics

The acute toxicity with *D. magna* was expressed as the effective concentration (EC_{50}-24 h), representing the percentage of concentration by volume (v/v) of the pharmaceutical wastewater that immobilizes 50% of the population. The EC_{50}-24 h and their limit of confidences were determined by the Hill model using Regtox (a macro software for Microsoft Excel version EV 7.0.6).

Data were first tested using Shapiro– Wilk tests for normal distribution, and the Levene test for homogeneity of variances. Then, the Wald-Wolfowitz test was used to detect significant differences between the means of the results of each treatment and control group. All statistical analyses were established at $p < 0.05$ using the software statistical program (STATISTICA version 6 for Windows, Statsoft, Tulsa, OK, USA).

In addition, the acute toxicity value was converted into Toxic Unit (TU_{50}-24 h) in order to explain the toxicity results according to the following equation:

$$TU50 = \frac{1}{EC50} X100 \quad \text{(Eq.1)}$$

In order to standardize the results, a hazard classification was developed by Persoone et al. (2003) in five classes as follows:

- No acute toxicity TU < 0.4.
- Slight acute toxicity 0.4 < TU < 1.
- Acute toxicity 1 ≤ TU < 10.
- High acute toxicity 10 ≤ TU < 100.
- Very high acute toxicity TU ≥ 1.

Results

Physicochemical results

The effluent quality of pharmaceutical wastewater is summarized in *Table 1*. As shown in *Table 1*, physicochemical parameters of pharmaceutical effluent show results as pH slightly acidic (6.60) with higher levels of EC (1065 µs cm^{-1}), TSS (3.44 mg/L), BOD_5 (110 mg/L), COD (280 ± 1124 mg/L), TN (5.38 mg/L) as well as sulfate (110 mg/L). However, we recorded a total absence of NH_4^+-N and TP in the wastewater (0 mg/L). All measured parameters are in compliance with general permissible limits discharges into surface waters recorded by Moroccan legislation (Moroccan Standard Discharges, 2023).

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In addition, analyses of heavy metals confirmed the highest iron concentration (1.8 mg/L). This result corroborates the finding of Kumari and Tripathi (2019). In their study, the wastewater generated by the pharmaceutical industry was heavily contained with iron (concentration ranged from 1.72 to 2.13 mg/L).

Table 1. *Physicochemical characterization of pharmaceutical effluent*

Parameters	Value	Moroccan standard discharges (2023)
pH	6.60	5.5-9.5
EC (µs cm^{-1})	1065	2700
TSS (mg/L)	3.44	100
COD (mg/L)	280	500
BOD_5 (mg/L)	110	100
Sulphate	110	600
TN (mg/L)	5.38	40
NH_4^+-N (mg/L)	0	NI
TP (mg/L)	0	15
Fe (mg/L)	1.8	5

NI: non-indicated

Toxicity test results of effluent from pharmaceutical process

Acute ecotoxicity test by D. magna

The percentages of immobilization and their confidence limits caused by pharmaceutical effluents on *D. magna* are presented in *Figure 1*. To validate the *D. magna* toxicity test, the number of immobile organisms should not exceed 10% in the controls.

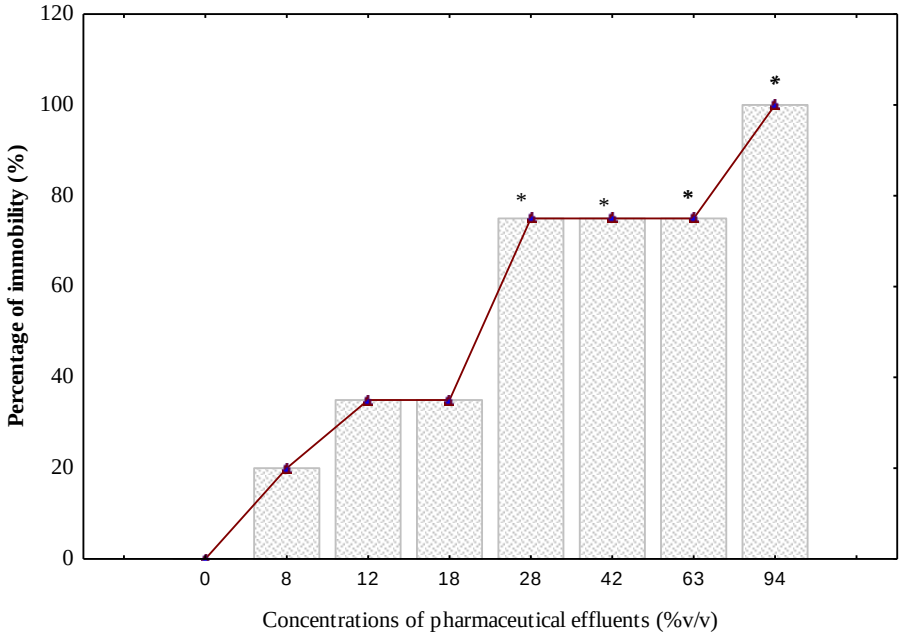

Figure 1. *Average percentage of immobilization effect and their confidence interval caused by pharmaceutical effluents on D. magna ($p < 0.05$). *Significantly different from the negative control (p-value < 0.05, Wald-Wolfwitz test)*

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After 24 h of exposure, the pharmaceutical industrial wastewater (PIWW) at the higher concentration tested (94.97% v/v) immobilizes 100% of neonates compared to the control. This total immobilization indicates that the effluent generated during the production process of pharmaceuticals contains toxic substances for *D. magn*a even if it was partially treated (*Fig. 1*). Up to 28.12% of exposure, the percentages of immobilization induced by this concentration achieve a statistically significant difference compared to the control**.** The percentages of immobility of daphnids varied from 40 to 100%.

The effective concentrations expressed as EC_{10}, EC_{25}, and EC_{50} after 24 h and their standards of variation (±SD) are given in *Table 2.*

Table 2. *EC_{10}, EC_{25} and EC_{50} and their ± SD after 24 h of exposition to PIWW*

Effective concentrations	EC_{10}-24 h	EC_{25}-24 h	EC_{50}-24 h
Values expressed as % v/v	6.63 ± 0.54	12.25 ± 1.74	22.65 ± 0.61

Chronic toxicity using D. magna

For chronic biotest, *D. magna* was exposed to seven nominal dilutions (0.067, 1, 1.25, 1.88, 2.82, 4.24, and 6.63% v/v). Effects on survival and different reproductive parameters (day to first brood, brood size, number of juveniles, and body length) of *D. magna* during 21 days of exposure are summarized in *Table 3.*

Table 3. *Longevity, day to first brood, brood size, number of neonates, and body length of D. magna exposed to concentrations of pharmaceutical effluent in 21 days (values are means ± SD)*

Concentration of effluent (%)	Longevity (days)	Day to first brood	Brood size	Number of neonates per female	Body length (mm)
0	21 ± 0.0	11 ± 1.0	24.25 ± 0.95	571 ± 0.31	4.12 ± 0.25
0.67	21 ± 0.0	11 ± 1.15	25.25 ± 2.5	566 ± 1.2	3.87 ± 0.25
1	21 ± 0.0	11.33 ± 0.57	26 ± 1.15	555 ± 1.11	4.12 ± 0.25
1.25	21 ± 0.0	11.33 ± 0.57	26.51 ± 1.0	567 ± 0.5	4.42 ± 0.25
1.88	21 ± 0.0	11.33 ± 0.57	29.25 ± 3.5	563 ± 0.2	4.75 ± 0.5
2.82	21 ± 0.0	11 ± 0.0	28.25 ± 0.71	574 ± 1.33	4.77 ± 0.0
4.24	21 ± 0.0	10 ± 0.0	35.25 ± 0.5*	730 ± 0.66*	5.1 ± 1.0*
6.63	21 ± 0.0	10 ± 0.0	37.15 ± 0.1*	770 ± 1.98*	5 ± 0.27*

*Significantly different from the negative control (p-value < 0.05, Wald-Wolfwitz test)

The long-term toxicity evaluation of PIWW on *D. magna* during 21 days shows a total absence of lethality for the overall concentrations tested (*Table 3*). As regards reproduction, a significant increase was detected in fecundity compared to the control (*Fig. 2*). The number of total neonates per individual shows a significant increase especially at the highest concentrations of 4.24% (730 ± 0.66; $p < 0.05$) and 6.63% (770 ± 1.98; $p < 0.05$) compared to the control (571 ± 0.31; $p < 0.05$). In addition, the brood size was higher 1.4 times at 4.24% (35.25 ± 0.5; $p < 0.05$) and 1.5 times at 6.63% (37.15 ± 0.1; $p < 0.05$) than the control (24.25 ± 0.95). However, there was no difference in the reproduction and brood size at the 95% confidence level between the

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control and concentrations less than 2.82%. Furthermore, Daphnia exposed to contaminated wastewater grew larger than the non-exposed population. The size of the body increased at higher concentrations of 4.24 and 6.63% v/v, and reached mean values of 5.1 ± 1.0 and 5 ± 0.27 compared to the control (4.12 ± 0.25).

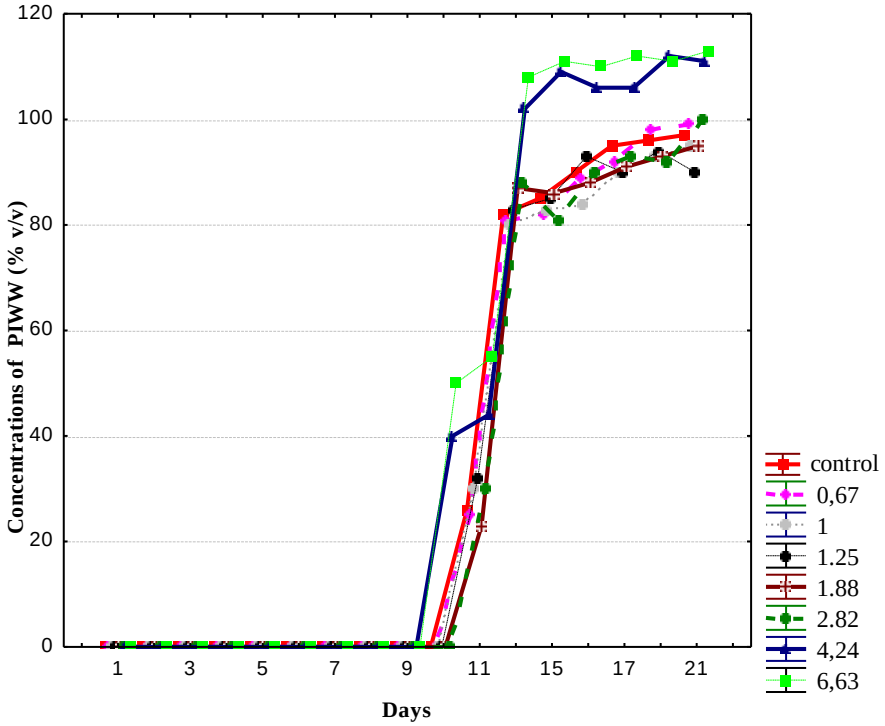

Figure 2. *Effect of pharmaceutical effluent on the number of neonates par female of D. magna during 21 days*

Discussion

Physicochemical parameters of pharmaceutical effluents

Many previous researchers have focused on the characterization of PIWW with regard to physicochemical properties, metals, and other toxic compounds (Rana et al., 2017; Kumari and Tripathi, 2019). In comparison with previous studies, our results are extremely different due to variations between manufacturing activities, raw materials as well as the formulation process employed (Mayabhate et al., 1988). However, the general pharmaceutical wastewater characterization existing in the literature was common to reveal higher concentrations of COD and nitrogenous. Cetecioglu et al. (2015) detected higher levels of COD (4.410 to 40.000 mg/L) in raw pharmaceutical wastewater. The frequent use of different nitrogen-containing organic and inorganic compounds as raw materials during the manufacturing process is the major origin of higher COD concentration in PIWW (Shi et al., 2017).

Acute toxicity with D. magna

Based on the results of *Table 2,* pharmaceutical effluent displays immobilizations towards *D. magna.* The EC_{50}-24 h reaches 22.65% ± 0.61. According to Persoone et al. (2003), our effluent belongs to the third class (TU_{50}-24 h = 4.41%) and it is categorized

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as acutely toxic to *D. magna*. This suggests that the PIWW in this study mainly contains high levels of toxic residual by-products generated during drug production. This finding revealed the importance of monitoring the toxic effect of PIWW using bioassay as a complementary tool to conventional parameters and highlighted the requirement for more effective treatments to depollute such complex effluents.

Compared to other studies, and despite the difference between the used samples and process our EC_{50}-24 h rejoined the acute toxicity using *D. magna* published by Tisler and Koncan (1999) (EC_{50}-24 h = 23.7%v/v). In addition, Larsson et al. (2007) demonstrated an immobilization effect on *D. magna* exposed to final effluents from drug manufacturers situated in Patancheru. In their study, the EC_{50}-48 h ranged from 6.7 to 7.2%v/v. The authors linked the acute toxicity to the abundance of nine target pharmaceuticals at nominal concentrations in the wastewater (concentrations ranging from 90 to 31,000 µg/L). Furthermore, Bielen et al. (2017) showed toxic effects expressed as the lowest ineffective dilution (LID) for which 10% of daphnids were immobile after 24 h of exposure to two PIWWs situated in Northern Croatia. In their assessment, the LID values exceed the permissible value for pharmaceutical effluent discharged into Chinese surface water (LID were 5 and 12% v/v, respectively). Cardoso et al. (2014) confirmed that the contamination of receiving aquatic environment in China and India is the final effluents from pharmaceutical facilities, in which popular antibiotics such as oxytetracycline and the hemostatic agent oxalic acid reached several mg/L (600 mg/L and 9100 mg/L).

Numerous authors have discussed the harmful effect of PIWW on water fleas and highlighted the major causes of acute toxicity. Hu et al. (2022) related harmful toxic effects on *D. magna* to the joint ecotoxicity posed by multicomponent mixtures, such as the residual antibiotics and organic toxic substances. Furthermore, Xue et al. (2022) showed a strong toxic effect of antibiotic production wastewater collected from Northern China on *D. magna* (TU reaching 625) and related this toxicity to residual toxic organic substances such as toluene, dichloromethane, tetramethyl hydrazine, and other raw materials. Kayode-Afolayan et al. (2022) showed that the presence of pharmaceutical actives substances and their residual by-products as well as recalcitrant substances such as organic matters (e.g., pharmaceutical actives), and inorganic nutrients (phosphates, nitrates, and sulphates) are the main source of toxic effect on immobilization of *D. magna*.

Chronic toxicity with D. magna

The information concerning the chronic impact of PIWWs on *D. magna* in the literature are very limited compared with the municipal wastewater effluents and chemical emergent compounds (e.g., pharmaceuticals). Tisler and Koncan (1999) showed chronic toxicity in *D. magna* exposed to pharmaceutical effluents. Compared with our study, the long-term toxicities in their study, are however changed in dose-response relations for survival, number of broods as well as the length of female adults. The authors linked the decrease in longevity, reproduction, and growth of daphnids after exposition to PIWWs to the presence of ammonia and zinc (used during the production process) in the final effluent. By contrast, our results corroborate previous observations, in which the long-term exposure of Daphnids to some human drugs stimulates the reproduction of *D. magna*. Svigruha et al. (2021) demonstrate that the exposition of *D. magna* to relevant environmental concentrations of progesterone stimulates the production of numbers of maximum eggs per individual at nominal concentrations of 1

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and 10 ng/L compared to the control. Likewise, different therapeutic classes such as antidepressants considered as non-hormonal drugs can affect the normal reproduction of *D. magna* by stimulation. In the literature, the fecundity increase using *D. magna* is usually reported with exposition to Fluoxetine (an antidepressant). Flaherty and Dodson (2005) demonstrated that chronic exposure to Fluoxetine at a nominal concentration of 36 μg/L elicits a significant increase in the reproduction of *D. magna*. A recent study carried out by Stremmel et al. (2023), showed the reproduction outputs of *D. magna* after 21 days were statistically different from the control and increased with higher concentrations of fluoxetine (100 μg/L). The number of juveniles reaching a mean value of 58.8 ± 4.6 compared to the control (46.4 ± 3.4). The authors linked the increase of reproduction to the first earlier brood of females in the contaminated treatment when compared to the control.

On the other hand, exposure to fluoxetine caused an increase in body size. Grzesiuk et al. (2023) demonstrate that Daphnids cultured with environmentally relevant levels of fluoxetine (360 ng/L) were on average 90 and 130 μm larger than individuals cultured without the treatment. The increase in body size in their study was explained by the increase in glucose and hyperglycemic hormonal levels in the hemolymph of several species of decapods, which are known to regulate reproduction and growth (Webster et al., 2012). In our study, the increased body length could be a positive effect of the presence of many toxic substances in PIWW at low concentrations (hormesis).

Based on the studies carried out in the literature, it may be that our PIWW contains high levels of endocrine disruptor as it directly affects reproduction. These findings impose more future analyses to confirm the main sources of this toxicity. Nevertheless, more research with multigenerational populations, and physiological and biochemical endpoints using the water flea should be performed to confirm the effect reported in this study.

Conclusion

Our study is the first one to assess the acute and chronic toxic effect of effluent from a pharmaceutical manufacturer in Morocco on *D. magna*. In conclusion, we found that the PIWW has a strong acute toxicity effect on *D. magna*. The exposition of PIWW has also affected the reproduction process by stimulation. In addition, the body length of adults showed a significant increase especially at higher concentrations compared to the control. This increase may be related to endocrine disruptor linked to drugs as it alters development and life cycle. More attention must focus on the optimization of more biochemical, physiological, and morphological to confirm the toxic effect of pharmaceutical wastewater.

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