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Evaluation of Ecotoxicity of Ibuprofen and Paracetamol on the Freshwater Green Microalgae "Pseudokirchneriella Subcapitata"

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ABSTRACT

The ibuprofen and paracetamol are amongst the most non-steroidal anti-inflammatory drugs (NSAIDs) used without prescription in the worldwide. The NSAIDs are frequently detected in receiving environments at trace levels. The sewage treatment plant effluents are considered an important continuous input of drug in aquatic ecosystems. This required the challenges on their potential treats on non-target organisms. The main objective of this study was to evaluate the potential ecotoxicity of ibuprofen and paracetamol on phytoplankton based on algal growth inhibition bioassay using 96-well microplates. A common freshwater green microalgae Pseudokirchneriella subcapitata was used due to its crucial position in aquatic chain food. The results showed that the exposure to ibuprofen elicits a chronic ecotoxicity on the growth of Pseudokirchneriella subcapitata where the Effective Median Concentration after 72 hours (72h-IC50) was 91.28 mg/L. However, paracetamol revealed no ecotoxicity on algal growth with a 72h-IC50 > 100 mg/L. In addition, the No Observed Effect Concentration of ibuprofen (NOEC) was (50 mg/L, $p < 0.005$), this value is still higher than the maximal concentration usually occurred in the aquatic environment.

KEYWORDS: ***NSAIDs, ibuprofen, paracetamol, growth inhibition, Pseudokirchneriella subcapitata.***

1. Introduction

Water is one of the biggest challenges of the 21st century. Currently, rising questions on the potential environmental risk of many anthropogenic substances. As one pharmaceuticals compounds (PCs) from human consumptions are belong the widespread emerging pollutants.

The major emission pathway of PCs into aquatic environment was the continuous discharge of sewage treatment plant effluents (STPEs) [1]. In Morocco as well as in the worldwide, the consumption of PCs increased namely via the self-medication. The most used therapeutic classes are the non-steroidal anti-inflammatory (NSAIDs), analgesic and antipyretic drugs such as ibuprofen and paracetamol [2, 3].

As a consequence to such a wide use, several studies reported the presence of ibuprofen and paracetamol (formerly known as acetaminophen) in aquatic matrices at trace levels in European, Asian and Northern American country. For example, Ginebreda and coworkers measured a concentration of ibuprofen up to 9.89 µg/L and a concentration of paracetamol up to 2.42 µg/L in the Llobregat River basin (Spain) [4]. In addition, Brun et al found paracetamol in Canadian receiving waters at median concentration of 0.005 µg/L, while ibuprofen was occurred at median concentration of 0.045 µg/L [5]. Moreover, Yan et al characterized the presence of both NSAIDs in

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the Yangtze River Estuary of China with concentrations up to 70 ng/L, and up to 10 ng/L of paracetamol and ibuprofen, respectively [6]. Recently, Gonzalez-Alonso et al confirmed the occurrence of PCs in surface Antarctic regions, where a highest concentration was occurred with paracetamol at 48.74 µg /L and ibuprofen at 10.05 µg /L [7]. In Morocco, the availability data about the occurrence of drugs in receiving environment are however, still lacking and any study was carried in this scope.

Currently, the ubiquity of ibuprofen and paracetamol in aquatic ecosystems highlights the concern of scientific community to its damages on the aquatic biota. Aguirre et al, showed a significant decrease in bioluminescence of freshwater bacteria *Vibrio fischeri* exposed to ibuprofen, in their study the IC_{50} after 15 min was 800 mg/L [8]. Therefore, Bang et al noticed that exposure to ibuprofen exhibited a remarkable lethality on the water flea *Daphnia magna (D.magna)* with a 48h-LC_{50} of 91.5 mg/L [9]. Likewise, Nunes et al concluded that the exposure to paracetamol displayed a toxicity on the bioluminescence of bacteria *Vibrio fisheri* (5min-IC_{50} was 92.2 mg/L). In the same study, paracetamol affected the mobility of *D.magna* (48-EC_{50}: 4.9 mg/L) and decreased numbers of fronds in the duckweed *Lemna minor* after 7 days (7d-EC_{50}: 429.9mg/L) [10].

By contrast, few studies were conducted to assess the ecotoxic potential of NSAIDs on phytoplankton. The most published works were especially pronounced for antibiotics. [11].

In Morocco, no previous study was carried to evaluate the ecotoxicity of ibuprofen and paracetamol on aquatic ecosystems. In view to enrich an additive knowledge, this first contribution aimed to evaluate the potential ecotoxicity effect of two NSAIDs (ibuprofen and paracetamol) on the growth of a freshwater green microalgae *P. subcapitata*. This is a common species widely used on the aquatic ecotoxicology due to its simple structure, ubiquity, rapid growth, short time response, and high sensitivity.

2. Materials and Methods

2.1. Tested Compounds

The ibuprofen and paracetamol selected in this study were purchased as salts from Kern Pharma (Spain). The stock solutions were prepared without co-solvent. Those solutions were then sterile-filtered using 0.2 µm filters and stored in the dark at 4 °C. Working solutions were prepared in 10 mL tube tests by combining the stock solutions with Environmental Canada medium [12]. The concentrations used for each drug were as flow (25, 50, 75, 100, 150, 200 mg/L).

2.2. Test Organism

The started culture of *P. subcapitata* was cultured in Environmental Canada medium under axenic conditions [12]. The culture was maintained in a growth chamber, which provides a standardized light using continuous fluorescent illumination cool-white having standard intensity 4000-6000 lux at the surface, at controlled temperature (24 ± 2°C.) and manual swirling (twice a day) after approximately four days (exponential growth).

2.3. Algal Growth Inhibition Bioassay using Microplates

The algal growth inhibition bioassay was run in polystyrene 96-well microplates according to Environmental Canada protocol [12]. Each well contained 200 µL of test solution, 10 µl of nutriment solution, and 10 µl of algal inoculum. For each drug, the treated cultures were performed in five replicates (well plates), whereas ten control groups were carried out. Finally, the microplates were sealed by parafilm to minimize evaporation of well contents. The tests were incubated in controlled room under 24-h uninterrupted fluorescent illumination (cool-white) having 4500-6000 Lux at the surface, at constant temperature (24 ± 2°C) and manual shaking (twice

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a day). The growth inhibition was quantified by absorbance at 450 nm after 24, 48, 72 and 96 hours using a microplate reader (BioTek ELx800).

3. Statistics

The Effective Median Concentration after 72 hours of exposure (72h-IC_{50}) and their limit of confidences were determined by Hill model using Regtox, a macro software for Microsoft Excel version EV 7.0.6 [13].

Data was first tested for normality and homogeneity of variances using Kolmogorov–Smirnov test and Levene's test, respectively. Then, One-way ANOVA of Kruskal walis was used to evaluate the significance difference between control groups and treatments. The No Observed Effect Concentration (NOEC) and the Low Observed Effect Concentration (LOEC) were determined using Dunnett's multiple comparison test. Statistical significance was established at $p < 0.05$ using the software statistical program (STATISTICA version 6 for Windows, Statsoft, Tulsa, OK, USA).

4. Results and Discussion

4.1. Effect of ibuprofen and paracetamol on algal growth of P.subcapitata

Algal growth of *P. subcapitata* exposed to serial concentrations of selected drugs (25, 50, 75, 100, 150, 200 mg/L) using 96-well microplates was recorded after 24h, 48h, 72h and 96h. As given in **Figure 1,** exposure to paracetamol showed no effect on growth of *P.subcapitata* at highest concentration tested (200mg/L), this is why it was no possible to define the 72h-IC_{50} **(Table 1).** By contrast, algal growth was inhibited when ibuprofen concentrations increased. Compared with the controls, a significant growth inhibition effect was observed at two high concentrations (150 mg/L) and (200 mg/L) of ibuprofen. The percentage of growth inhibition was (66%). Whereas a stimulatory effect (-25%) was promoted at concentrations lower than (50 mg/L). This stimulation may be due to hormesis phenomenon (a low dose stimulation).

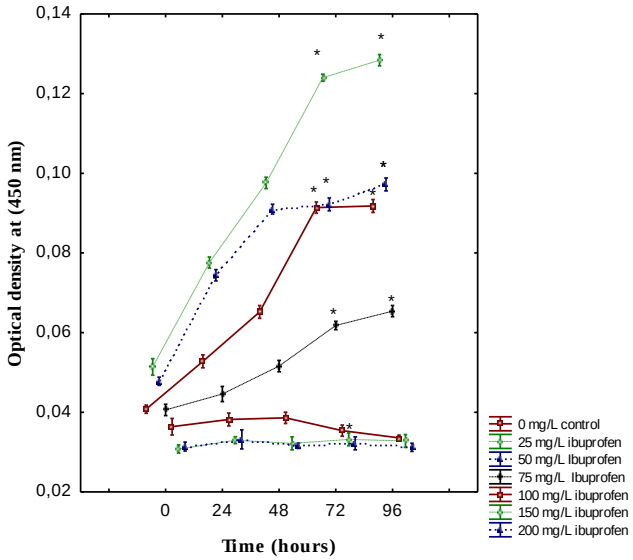

Figure 1. Growth curves of *P. subcapitata* exposed to different concentrations of ibuprofen after 24h, 48h, 72h, and 96h. Each point is the mean of five replicates. Error bares indicate the standard deviation.

* Significant difference with control at $p < 0.05$.

The effect concentrations of ibuprofen and paracetamol expressed as EC_{10}, EC_{25} and EC_{50} (mg/L) and their 95% confidence limits after 72 hours are presented in **Table 1.** A difference in sensitivity to *P. subcapitata* against selected NSAIDs was observed; exposure to paracetamol revealed a 72h-IC_{50} >100 mg/, while exposure to ibuprofen gave a slight higher response to *P.subcapitata* (72h-IC_{50} = 91.28 mg/L).

According to the European Directive 93/67/EEC [14]. The ecotoxicity of contaminants was classified in different risk classes based on their measured effective concentration values (IC_{50}) as follow: an IC_{50} <1 mg/L would be classified as ''very toxic to aquatic organisms''; $1 < IC_{50} < 10$ mg/L ''toxic to aquatic organisms'', $11 < IC_{50} < 100$ mg/L ''harmful to aquatic organisms, and chemicals with $IC_{50} > 100$ mg/L would not be classified. Within this scheme, the ibuprofen (72h-IC_{50} = 91.28 mg/L) in our study could be categorized as a harmful emerging substance to

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P.subcapitata. While paracetamol (72h-IC_{50} > 100 mg/L) would not be classified.

Table 1. The effective dose-response parameters (mg/L) and their 95% confidence limits after 72h.

drug	IC_{10}	IC_{25}	IC_{50}	NOEC	LOEC
ibuprofen	34.14 (19.51 -49.79)	55.58 (39.06 -72.04)	91.28 (75.70-107.49)	50	25
paracetamol	-	-	>100	-	-

As previously mentioned by Blaise et al [15], exposure of *P.subcapitata* to ibuprofen using microplates showed a harmful effect. In their work the 72h-IC_{50} was 90.5 mg/L. This result is within those obtained in our study. By contrast, testing of ibuprofen using classical methods show a lower sensitivity of *P.subcapitata* with others researchers. According to Gonzalez-Naranjo and Boltes [16], ibuprofen was classified as not harmful to *P.subcapitata* with a 72h-IC_{50} of 232 mg/L. Similarly, a lesser sensitivity to *P.subcapitata* using vials was observed by Aguirre-Martinez et al [8], whose 72h-IC_{10} value was 40.7 mg/L compared to 72h-IC_{10} (34.14 mg/L) obtained in our study. The origin of the discrepancy in responses of *P.subcapitata* may be related to difference in protocols tested. The obtained NOEC value of ibuprofen in our study (50 mg/L, $p < 0.05$, Table 1) was still higher than the occurred concentrations usually detected in freshwaters [4-7]. This suggests a lower environmental risk of ibuprofen in aquatic ecosystems.

Moreover, in this study, the results revealed that exposure to paracetamol was no toxic on growth rate of *P.subcapitata* (72h-IC_{50} >100 mg/L). This result met the finding of two recent works carried by Mingez et al and Nunes et al [10, 17]. This suggests that microalgae is not a target organism to paracetamol.

4.2. Overview of the ecotoxicity of ibuprofen and paracetamol on green microalgae

Various studies have reported the effect of ibuprofen and paracetamol on growth of phytoplankton. **Table 2,** summarizes an overview on reported data in the literature. In general, a difference in tolerance against taxonomical groups of green microalgae toward ibuprofen and paracetamol was observed. As mentioned in **Table 2,** we can observed that algal species belong genus "*Scenedesmus"* appears more sensitive to both drugs while *P.subcapitata* was the last sensitive.

In fact, *Scenedesmus rubescens* displayed a significant toxicity to ibuprofen (72h-IC_{50} = 1 mg/L) while *Senedesmus subscapitata* was the most sensitive to paracetamol (72h-IC_{50} = 134 mg/L) [18,19].

This suggests that the sensitivity of microalgae to selected drugs is highly species dependent. The outcome of IC_{50} outlined in **Table 2** demonstrated that the ibuprofen was considered harmful to all tested green microalgae (in the most cases: 11< 72h-IC_{50} <100 mg/L) while the ecotoxicity of paracetamol cannot be classified. Krienitz et al [20] explained the origin of the weak sensitiveness of green microalgae *Nannochloropsis Limneticaa* to PCs including ibuprofen to its strong cells walls, which would be prevent the ingestion of drugs.

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Table 2. Overview of the effect data of ibuprofen and paracetamol on green microalgae reported in the literature.

Drug	Algal species	IC(mg/L)	endpoints	References
ibuprofen	*Isochrysis galbana*	74.7	72h-IC_{50}	[8]
	Pseudokirchneriella subcapitata	40.7	72h-IC_{10}	[8]
	Pseudokirchneriella subcapitata	232.64	72h-IC_{50}	[16]
	Scenedesmus abudans	1.170	72h-IC_{50}	[21]
	Desmodemus subspicatus	315	72h-IC_{50}	[22]
	Scenedesmus subspicatus	342.2	72h-IC_{50}	[23]
	Pseudokirchneriella subcapitata	2.3	96h-IC_{50}	[24]
	Scenedesmus rubescens	1	72h-IC_{50}	[18]
	Chlorella vulgaris	86.43	72h-IC_{50}	[265]
	Pseudokirchneriella subcapitata	90.5	72h-IC_{50}	[15]
	Pseudokirchneriella subcapitata	360	96h-IC_{50}	[26]
	Desmodesmus subcapicatus	315	72h-IC_{50}	[27]
	Pseudokirchneriella subcapitata	No effet	72h-IC_{50}	[5]
	Pseudokichneriella subcapitata	91.28	72h-IC_{50}	Our study
paracetamol	*Pseudokirchnriella subcapitata*	2300	96h-IC_{50}	[22]
	Pseudokirchneriella subcapitata	>100	72h-IC_{50}	[10]
	Senedesmus subscapitata	134	72h-IC_{50}	[19]
	Pseudokirchneriella subcapitata	317.4	72h-IC_{50}	[24]
	Pseudokirchneriella subcapitata	>100	72h-IC_{50}	Our study

However, the lesser sensitivity of eukaryotic micro algae to ibuprofen does not exclude its effect on others target endpoints. In fact, the weak toxicity of ibuprofen on algal growth rate suggests its effect on vitality, pigment amounts, photosynthetic, cellular activity as well as morphological or physiological alterations. Indeed, the finding of Moro et al [28] confirmed functional alterations on photosynthetic capacity at relevant concentration 1000 µg/L (a decrease in chlorophyll and increase of caretoinoides contents) on green microalgae Scenedesmus rubescens under ibuprofen exposure. Likwise, Vanini et al [29] indicated an ecotoxic effect on chloroplasts of P.subcapitata in presence of therapeutic mixtures includingibuprofen.

Hence, the inclusion of further biomarkers in ecotoxicological assessment of selected drugs to *P.subcapitata* are recommended to understand their mode of action especially at nominal environmental doses.

5. Conclusions

This preliminary study demonstrated that the sensitivity of *P.subcapitata* to selected drugs is strongly compounds and species dependent. Exposure to ibuprofen was more toxic on growth of *P.subcapitata* as compared to paracetamol exposure, where the Effective Median Concentrations after 72 hours were 72h-IC_{50}: 91.28 mg/L and 72h-IC_{50} >100 mg/L, respectively.

Those effects data are still higher than the usually occurred concentrations reported in aquatic ecosystems in the literature. This suggests that the growth inhibition bioassay using the freshwater *P.subcapitata* is not suitable for ecotoxicity evaluation of ibuprofen and paracetamol.

Further experiments on other test organisms belong to algal or other aquatic species are necessary to fully confirm the ecotoxicity of those components in the receiving waters. In addition, the effect data of specific biomarkers of ibuprofen

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and paracetamol on the *P.subcapitata* are almost lacking and needed a more mater of active researches.

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