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Toxicological effects of drugs (Diclofenac, Ibuprofen, mixture) and Hormesis on a non-target organism: *Paramecium sp*

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Abstract
The exposure to pharmaceutical residues is inevitable, they are present in all ecosystems, particularly Non Steroidal Anti-Inflammatory Drugs and their mixtures. In fact, the impact studies of these compounds on microorganisms are infrequent. In this context, the toxicity evaluation of Diclofenac, Ibuprofen and mixture, is undertaken on *Paramecium sp*, researching the effects of these xenobiotics on cellular growth and respiratory metabolism. The results show that cells exposure to low concentrations of Ibuprofen (1 and 10 µg/L), induces a insignificant stimulation, of cell growth and respiratory metabolism, in contrast to cells treated with Diclofenac, where an inhibition of cell growth is observed. The two xenobiotics mixture treatments show a significant toxicity in the presence of Diclofenac. The specific behavior of *Paramecia* with low concentrations of Ibuprofen may sugget a hormetic dose-response curve, expressed by an increase in the two measured physiological processes, cell growth and respiration.

Keywords: Non Steroidal Anti-Inflammatory Drugs, toxicity, *Paramecium sp*, growth kinetics, respiratory metabolism

1. Introduction
The use of chemicals, especially medicines and other pharmaceuticals in the world is significant. The presence of pharmaceutical residues in the environment creates a major problem for human's health, making this, issue a recent investigation subject [1]. Contrary to most other xenobiotics, pharmaceutical residues products have an important peculiarity, they can interact together and thus, act at very low concentrations (of around 1 ng/L) [2]. Among many drugs, Non Steroidal Anti-Inflammatory Drugs (NSAIDs) constitute an important group, besides the fact that their sale is not subject to medical prescription [3], which is the case in Algeria. In addition, once these compounds get expired, they are directly dumped into the environment, further increasing the danger of contamination even after expiration [4, 5]. Pharmaceuticals consumed by humans and animals can be either completely, partially or not metabolized at all, which generates either intact molecules or metabolites [6]. This represents a real danger, especially that these discharges into the environment are continuous. In the literature, many research studies reveal the toxicity of these substances in sewage [7]. Gibson *et al*. [8] have recorded the presence of high concentrations of Diclofenac in wastewater of the Tula region in Mexico. Fent *et al*. [5] have reported the presence of NSAIDs in domestic drinking water. Hussein *et al*. [9] have studied that the presence of Paracetamol doesn't seem to have adverse effects on plankton. In this context, NSAIDs may have different toxicological profiles because of their chemical composition, their internal (same molecules) or external (different molecules) interactions. All these factors are able to influence the dose-response relationship at many biological phenomena. Therefore, hormesis is defined as the relationship between dose and response, characterized by stimulation at low concentrations and inhibiting at high concentrations [10]. This phenomenon is generally described as a dose-response curve inverted "U" or "β". The hormesis is observed in many microorganisms, plants and other various animal groups. In the order to explore the effects of the two NSAIDs (Diclofenac, Ibuprofen and their mixture) on a food chain link, we work on the possible highlighting of the hormetic dose-response on cell growth and respiratory metabolism of *Paramecium sp* in laboratory conditions.

2. Materials and Methods

2.1 Chemical products

Both xenobiotics used for this work are Diclofenac and Ibuprofen, they belong to NSAIDs family, and were obtained from Sigma-Aldrich.

2.2 Culture and cell growth

The *Paramecium sp* are eukaryotic protozoa, well known by biologists, and frequently used to study the effects of water pollution [11]. They are cultivated inside plant infusion enriched with *Saccharomyces cerevisiae*, to encourage growth, and incubated at 27 ± 3 °C away from light. The cell culture will grow exponentially until attain a stationary phase [12].

2.3 Toxicity tests

After reaching this stationary phase, the paramecia were exposed to increasing concentrations of Diclofenac and Ibuprofen (1, 10, 100 and 1000 µg/L) and their mixtures (1 and 1000 µg/L).

2.4 Growth kinetics

Growth kinetics are made by counting cells using a manual counter under an optical microscope (x 10) at 24, 48, 72 and 96h [13].

2.5 Respiratory activity

Oxygen consumption is measured in stationary phase, it is made with a Hansatech oxygen electrode [14].

2.6 Statistical analyses

All experiments were repeated three times, results are presented as average ± standard error (SE) values. Statistical analysis used for comparison between the control and treated values are: one-way ANOVA, the Paired T test for dependent samples data which are normally distributed, and the Wilcoxon test for non normal data. (α- level for significant differences is at $P \leq 0.05$, and for high significant differences is at $P \leq 0.01$).

3. Results

Effects of Diclofenac and Ibuprofen on the growth of *Paramecium sp* cells.

Fig. 1(A.B.C.D.E). show the results of the effects of both NSAIDs and their mixtures on the cell growth of *Paramecium sp*.

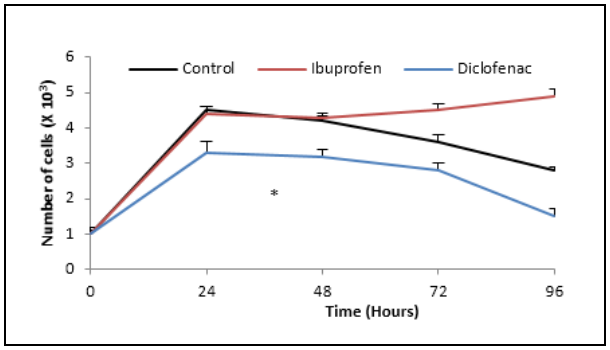

Fig 1A: Effect of xenobiotic Diclofenac and Ibuprofen (1 µg/L) on Cell number of *Paramecium sp*. Each data point represents the mean of three dependent assays ± standard error. *: Significant values compared with control $P \leq 0.05$.

Low concentrations (1 µg/L and 10 µg/L) act differently on paramecia. When Ibuprofen seems to stimulate, even not significantly, growth starting at 48h, with a progression of 75% with 1 µg/L and 35% with 10 µg/L at 96h, Diclofenac, slows the growth significantly (P = 0.02 and 0.01 respectively) of about 55% at 96h compared to controls Fig.1. (A, B). Against, high concentrations (100 µg/L and 1 mg/L) act similarly, they inhibit significantly ($P \leq 0.05$) the growth of 11% with 100 µg/L and 61% with 1 mg/L for Ibuprofen and about 60% for Diclofenac Fig. 1. (C, D).

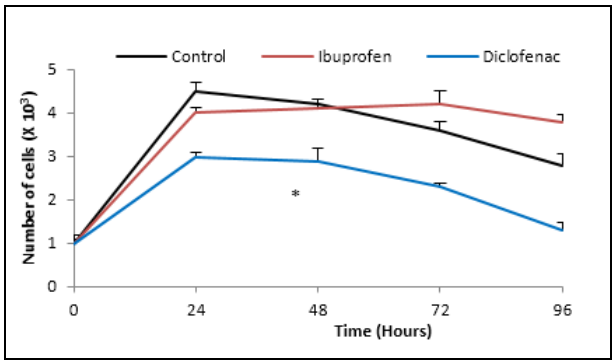

Fig 1B: Effect of xenobiotic Diclofenac and Ibuprofen (10 µg/L) on Cell number of *Paramecium sp*. Each data point represents the mean of three dependent assays ± standard error. *: Significant values compared with control $P \leq 0.05$.

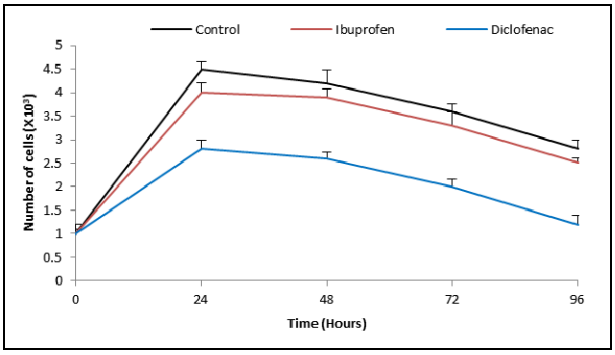

Fig 1C : Effect of xenobiotic Diclofenac and Ibuprofen (100 µg/L) on Cell number of *Paramecium sp*. Each data point represents the mean of three dependent assays ± standard error. Significant values compared with control $P \leq 0.05$.

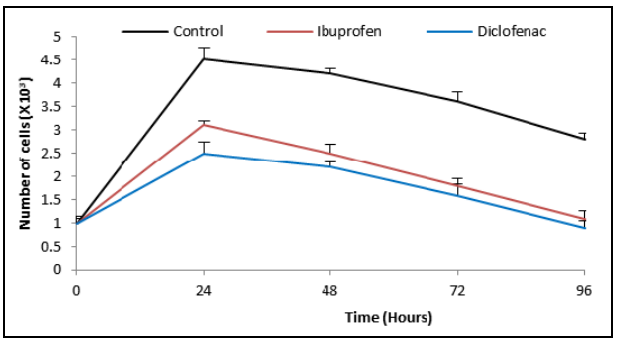

Fig 1D: Effect of xenobiotic Diclofenac and Ibuprofen (1 mg/L) on Cell number of *Paramecium sp*. Each data point represents the mean of three dependent assays ± standard error. Significant values compared with control $P \leq 0.05$.

Fig. 1E. shows the effects of the two NSAIDs mixtures on cell growth of *Paramecium sp*. There is a significative growth slowdown with observed inhibitions with all tested concentrations ($P \leq 0.05$), the presence of Diclofenac alone can reverse the stimulatory effect of Ibuprofen at low

concentrations. The high concentration of Diclofenac or Ibuprofen or both (1 mg/L) leads to a inhibition of growth up to 60% Fig. 1E., this confirms the frequently unexplained effect of different mixtures that are formed and accumulated in the environment, in direct contact with non-target organisms.

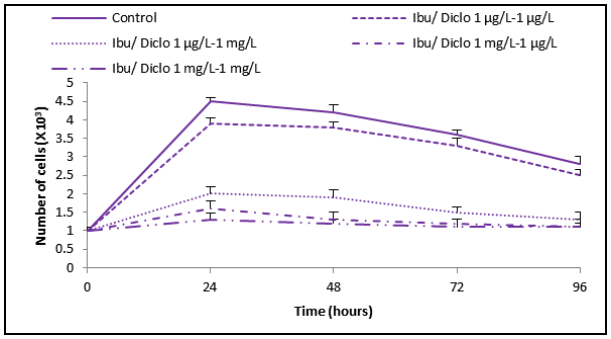

Fig 1E: Effect of Ibuprofen / Diclofenac mixtures on Cell number of *Paramecium sp*. Each data point represents the mean of three dependent assays ± standard error. Values compared with control are significant with $P \leq 0.05$.

Effects of Diclofenac and Ibuprofen on the respiratory metabolism of *Paramecium sp* cells.

Fig. 2 (A.B.C). show the results of the effects of both NSAIDs and their mixtures on the respiratory metabolism of *Paramecium sp*.

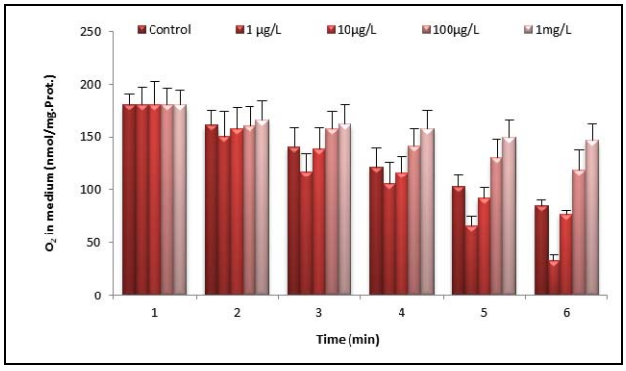

Fig 2A: Effect of Ibuprofen on respiratory metabolism of *Paramecium sp*. Each data point represents the mean of three dependent assays ± standard error.

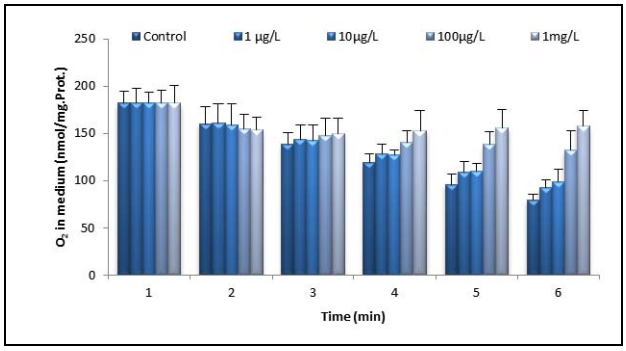

Fig 2B: Effect of Diclofenac on respiratory metabolism of *Paramecium sp*. Each data point represents the mean of three dependent assays ± standard error.

Fig. 2A. Shows that Ibuprofen is in two different ways according to the used concentrations. In the presence of low concentrations (1 and 10 µg/L), treated cells breathe strongly and oxygen of the medium decreases, indicating cells hyperactivity, probably due to the paramecia defense mechanisms Fig. 2A. Meanwhile, high concentrations (100 µg/L and 1 mg/L) affect the respiratory metabolism, expressing the inhibitory effect of Ibuprofen. Fig. 2B. shows that Diclofenac inhibits respiration as function of time and this, from the lowest concentration tested.

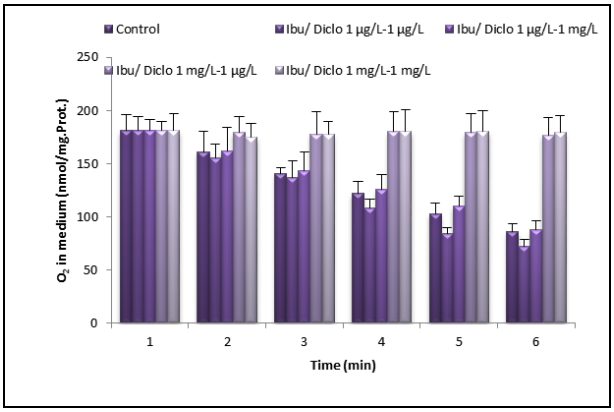

Fig 2C: Effect of Ibuprofen / Diclofenac mixtures on respiratory metabolism of *Paramecium sp*. Each data point represents the mean of three dependent assays ± standard error. Values Higly significants with $P \leq 0.01$.

Fig. 2C. shows the effects of two NSAIDs mixtures on respiration of *Paramecium sp* (P = 0.003). First, there is a slight positive effect recorded to low concentrations of two NSAIDs (1 µg/L), followed by a start inhibition of respiration observed at the concentration of 1 mg/L of Diclofenac. At last, we observe a cessation of cells respiratory metabolism treated with high concentrations.

4. Discussion

In this work, we noted two types of responses for the two NSAIDs studied and their mixture, on cell growth and respiratory metabolism of *Paramecium sp*. The first observation is that cells exposure to low concentrations of Ibuprofen (1-10 µg/L), seems to induce a stimulation of growth and respiration. Beyond all these concentrations, a significant inhibition of the cellular growth is observed. Furthermore, the cells exposure to different concentrations of Diclofenac causes high toxicity, reflected in a sharp reduction in cell growth and cessation of breathing at different concentrations. Generally, it is accepted that both NSAIDs studied are used in humans to relieve pain and suppress inflammation [15]. Their mode of action consists in inhibiting an enzyme (COX) that catalyzes the biosynthesis of prostaglandins [16]. Thus, in birds for example, prostaglandins participate in the biosynthesis of shells, the inhibition of COX causes eggshell thinning which results in reduction of hatching [17]. Pomati *et al*. [18] have studied that 1 mg/L of Ibuprofen reduced the growth of duckweed *Lemna minor* of 25%, while conversely, the growth of cyanobacteria *Synechocystis sp* was stimulated to 10 µg/L. Han Guk *et al*. [19] have compared chronic toxicity of Diclofenac and Ibuprofen, Diclofenac was more toxic than Ibuprofen. Indeed, a concentration of 40 mg/L of Diclofenac was enough to achieve a 100% mortality in *Daphnia magna* unlike 80mg/L concentration for Ibuprofen. Cleuvers [4] has demonstrated the high toxicity of a mixture of Diclofenac, Ibuprofen, Naproxen and Acetylsalicylic Acid on algae and daphnia. This toxicity was as high or higher than the toxicity

of each molecule used alone. Gómez-Oliván *et al*. [20] have demonstrated that different NSAIDs, isolated and binary mixtures, induce oxidative stress on *Hyalella azteca.* A decrease in the activity of the freshwater amphipod *Gammarus pulex* was also noticed when in contact with Ibuprofen at concentrations of 1 and 10 ng/L [21]. Nieto *et al*. [22] have studied that Diclofenac may produce respiratory deficiencies in the freshwater shrimp, *Atyaephyra desmarestii* under certain temperature and water oxygenation conditions. However, after chronic exposure, several authors observed histopathological effects in trout species at relatively low concentrations of Diclofenac, around 1 to 5 µg/L [23, 24]. Moreover, a study conducted *in vitro* on a bivalve mollusc gives an LC_{50} of 178 µg/L for Diclofenac and 1312 µg/L for Ibuprofen, thus demonstrating the seriousness of Diclofenac toxicity [25]. In more recent work, Boulassel [26] shows a toxic effect of Ibuprofen in high concentrations resulting in a slowdown of cell growth, presence of cellular defects, decreased number of vacuoles and inhibition of paramecia respiration. Trombini *et al*. [27] they have found that after 48 hours exposure of neonate nauplii (<24 h-old) of the harpacticoid copepod *Tisbe battagliai*, that Diclofénac was the most toxic compound with a LC_{50} 5 to 7 times lower than LC_{50} value for Paracetamol, Carbamazepine and Ibuprofen. The observations made in our work on the exposure of cells to mixtures of two NSAIDs show a global effect representing the sum of the effects of both xenobiotics, and this is due to different interactions and other synegies that may occur [28]. Studies of combinations give an antagonist effect in 59% of cases, additive effect in 37% and only 4% of synergy effect [29]. Thus, the observed effects following exposure to both individual NSAIDs and their mixture are very different [30, 4]. After comparing the different results obtained with all concentrations used for each compound and for the mixtures, it is clear that the low concentrations of Ibuprofen are the origin of a cell growth and a respiratory metabolism stimulation. In fact, this seems to be the induction of a hormetic response specific to measured parameters, and directly related to the cell model used known *Paramecium sp*.

5. Conclusion

The review of the toxicological effects of Diclofenac and Ibuprofen on *Paramecium sp,* shows that, contrary to high concentrations, at lower, Ibuprofen doesn't affect Paramecia significantly, although, a possible hormetic response remains to explore. Nevertheless, the toxicity results obtained with Diclofenac and mixtures are not sufficient to draw definitive conclusions. Therefore, research is required with a larger number of samples, and should be focused on the use of a wide range of concentrations with temporal evaluations.

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