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Effect of Pharmaceutical Compounds (Diclofenac, Ibuprofen, and Erythromycin) on the Heterotrophic Behaviors of Biomass of a Membrane Bioreactor to Treat Urban Wastewater

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Abstract: The increasing prevalence of pharmaceutical substances in wastewater is emerging as a pressing ecological issue on a global scale. The purpose of this study was to evaluate the biological influence of pharmaceutical compounds on the heterotrophic biomass residing in a membrane bioreactor. The study examined the way microorganisms react to antibiotic and anti-inflammatory compounds, with the goal of proactively tackling potential issues and developing solutions that may emerge withing wastewater treatment plant bioreactors. Respirometric tests were carried out to determine the kinetic response of the heterotrophic biomass. The same study was carried out in the steady state of the plant under different conditions of hydraulic retention times (6 and 12 h) and biomass concentration (2888 ± 371 mg/L to 7477 ± 869 mg/L). A response surface statistical analysis was applied to determine the effect of the variables on the rate of substrate degradation for organic matter removal and the growth rate of net heterotrophic biomass. The results show that the biological response of the biomass is concerned when exposed to a combination of pharmaceutical substances such as ibuprofen, diclofenac, and erythromycin, in four cycles of operation at 16 varying concentrations of pharmaceuticals in each cycle. This suggests the presence of a synergistic effect among these pharmaceuticals, leading to a noticeable slower kinetic response in the biomass.

Keywords: pharmaceutical compounds; kinetic modelling; heterotrophic biomass; wastewater treatment; membrane bioreactor

Citation: Antiñolo Bermúdez, L.; Díaz Mendoza, V.; Poyatos Capilla, J.M.; Muñío Martínez, M.d.M.; Martín Pascual, J. Effect of Pharmaceutical Compounds (Diclofenac, Ibuprofen, and Erythromycin) on the Heterotrophic Behaviors of Biomass of a Membrane Bioreactor to Treat Urban Wastewater. *Environments* **2023**, *10*, 198. https://doi.org/10.3390/environments10120198

Academic Editors: Zhen Wei, Yueping Bao and Wenlu Li

Received: 28 September 2023
Revised: 16 November 2023
Accepted: 20 November 2023
Published: 22 November 2023

1. Introduction

The human population is growing steadily, and this rapid rise has increased urbanization significantly over the last few decades [1]. The continuously growing water demand accompanied by climate change and dwindling energy resources has resulted in a global challenge of water scarcity [2]. Properly managing and treating wastewater is a global challenge because, if it is not properly treated, it can lead to the release of pollutants into the natural environment. The profound toxicity and resistance to degradation exhibited by persistent hazardous chemicals render water pollution a menace to environmental stability [3]. These pollutants are the so-called compounds of emerging concern (CEC), which include pharmaceuticals, pesticides, hormones, personal care products, and illicit drugs. They have garnered substantial interest owing to their extensive production, diverse array of applications, and frequent identification in both surface and groundwater sources [4]. Among emerging pollutants, pharmaceuticals are a unique group structurally designed to maximize their intrinsic biological activity at low concentrations and developed to induce a continued action in biological systems [5].

The European Union, through Directive 2008/105/EC, stipulates the creation of an observation list of substances for the purpose of systematic monitoring at the European level.

When considering the United States, the Environmental Protection Agency (EPA, https://www.epa.gov/ccl, last accessed 10 October 2022) publishes a Candidates Contaminants List. In the case of Japan, the control of these pollutants is carried out by the Ministry of Health. Countries such as Canada and Australia also have their own legislation. In the context of China, the Ministry of Environmental Protection has formulated a strategy designed for the control and prevention of environmental risks of chemical substances, including pharmaceuticals. While they may be present in water at concentrations spanning from ng/L to µg/L, their removal is imperative due to their persistence, as these compounds have the potential to exert a substantial impact on public health and the environment [6]. Antibiotics are being used intensively for humans and livestock worldwide and have led to the presence of antibiotic-resistant bacteria and antibiotic-resistant genes in the environment [7]. These bacteria have the capacity to transfer their genetic material to aquatic microorganisms, which likewise possess genes conferring resistance [8,9]. In addition, many antibiotic compounds, such as erythromycin, can be discharged into the sewage system and, as a consequence, affect the microbial community and generate resistant bacterial strains [6].

The effluent discharges of wastewater treatment plants (WWTPs) are considered the main route of entry of these pollutants into the environment [10,11]. The occurrence of a broad variety of micropollutants in municipal wastewater makes WWTPs the primary barriers against their spread in the environment [12]. However, the inability to effect complete removal of CECs from WWTPs poses a potential risk to aquatic organisms and public health [13], as the existing treatment facilities are designed to remove biodegradable organics and nutrients (the discharge of nutrients such as nitrogen and phosphorus in high concentration, causing associated problems of eutrophication [14]) and cannot effectively eliminate these recalcitrant chemicals, leading to a considerable discharge of pharmaceuticals into aquatic environments [15]. Despite their relatively low concentrations, these compounds pose deleterious ecological effects, as the persistence of antibiotics in the environment has been linked to the emergence of antibiotic-resistant bacteria and the spread of resistance genes in the environment [16]. In addition, metabolites may arise during the degradation of these pharmaceutical compounds, which in some cases may be more toxic than the original compound [17]. An investigation conducted on a European scale identified a cumulative sum of 477 pharmaceutical substances and 66 metabolites. Of these substances, 243 pharmaceuticals and 41 metabolites were detected at concentrations over the detection limit [18]. Among these compounds, diclofenac and ibuprofen, as well as their metabolites 4-acetamidoantipyrine and 4-formylaminoantipyrine, were frequently detected in more than 28 European states. Among the predominant antibiotics, erythromycin was frequently detected in surface waters. [18]. Additional research has proposed potential hazards associated with erythromycin in Asia [19,20], as well as in the surface waters of the Middle East and North Africa [21]. Specifically in China, diclofenac, ibuprofen, and erythromycin were identified as compounds with a moderate to high risk to aquatic organisms [22]. Ibuprofen and its metabolites 2-Hydroxy ibuprofen and Carboxy ibuprofen were detected in river sediments [23,24]. Diclofenac and its metabolite 4-hydroxy diclofenac were also detected in river sediments [25], as well as 5-hydroxydiclofenac and its metabolite p-Benzoquinone imine of 5-hydroxydiclofenac [23]. Erythromycin was detected in crucian carp after exposure to the antibiotic, as well as its metabolites dehydration-didemethylated-erythromycin and dehydration-descladinose-erythromycin [26,27]. Several studies have highlighted the associated chronic effects caused by the toxicity of compounds to evaluate specific environmental risks [28]. Diclofenac caused mortality after 14 days of exposure to earthworm *Eisenia fetida* [29] and immobilization at 48 h in *Daphnia magna* in a marine freshwater environmental assessment in northwest France [30]. In the case of erythromycin, it caused cell disintegration in *chlorella vulgaris* and *Ankestrodesmus falcatus* after 24 h of exposure [31]. Ibuprofen caused growth inhibition in *chlorella vulgaris* after 48 h [32] and in *Navicula* sp. after 10 days [33], as well as mortality in *Eisenia fetida* after 14 days [29]. The treatment of wastewater containing pharmaceuticals is usually quite complex due to the presence of solvents or organic compounds that are often non-biodegradable and/or

toxic for microorganisms in biological treatments [34]. Hence, it becomes imperative to investigate efficient approaches for the elimination of pharmaceutical compounds from water bodies [35].

Membrane bioreactor (MBR) technology is being increasingly adopted by numerous nations due to its advantages in terms of a compact design, high quality of treated water, and simplified operational procedures [36]. It integrates biological treatment with membrane filtration to produce a clarified effluent [37,38]. In addition, membrane technology, which encompasses a number of characteristic separation processes, offers a more holistic solution and makes provisions for treatments that deal with both salinity effect and the particularity of various pollutant characteristics [39]. Despite the problems of membrane fouling and high energy costs, it is an efficient technology with pollutant removal efficiencies close to 100% in some cases [40]. Among the pollutants, not only emerging contaminant compounds are covered, but this technology can also be applied to various insoluble (i.e., oils) and soluble (i.e., heavy metals) contaminants [41]. For those types of pollutants (such as oil or petroleum) more specific to industrial and urban wastewater, nanofibrous membranes with visible light-induced self-cleaning capabilities are being developed, with properties such as high porosity, super wettability, and low oil adhesion [42]. New types of composite hydrogel membranes of chitosan (CS) and nanohydrotalcite (LDHs) in a polyvinyl alcohol (PVA) hydrogel (named PVA-CS-LDHS) are also under development for oil–water separation and metal cation removal [43]. Plant control with detailed characterization of the influent and effluent streams and the concentration of the biomass in the biological reactor is essential for efficient treatment [44]. It is equally important to ascertain the response of biomass in the presence of contaminants, and to this end respirometry has demonstrated its utility and effectiveness as a method for determining the kinetic parameters of biomass. It furnishes insights into substrate elimination and biomass growth [45,46]. Consequently, this investigation endeavors to analyze the response of microorganisms to antibiotic and anti-inflammatory pharmaceuticals, with the objective of preemptively addressing potential challenges and devising solutions that might arise within the bioreactors of WWTPs in the event of seasonal or accidental discharges.

The aim is to investigate the influence of ibuprofen, diclofenac, and erythromycin on the behavior of heterotrophic biomass within a membrane bioreactor receiving urban wastewater and, concurrently, comprehend the biomass response to pharmaceutical discharges in the wastewater treatment plant, thereby facilitating the development of effective removal strategies for these substances from the treated effluent. These pharmaceuticals were chosen because they are present on the EU monitoring list, and also due to their high detectability in aquatic media. They were also chosen by taking into consideration the compound's solubility, and notably, one of them possessed antibiotic properties. For this purpose, respirometric tests were carried out to evaluate the effect of these pharmaceutical compounds, individually and as a whole, on the kinetic modelling and to evaluate the effect under different hydraulic retention time (HRT) and mixed liquor suspended solid (MLSS) operating conditions.

2. Materials and Methods

2.1. Description of the Pilot Plant of Membrane Bioreactor

The semi-technical pilot scale plant with MBR technology is located at Los Vados WWTP in Granada (Spain) and is fed with real urban wastewater. The pilot plant (Figure 1) consists of a mixing tank with mechanical agitation and a rectangular bioreactor (85 L) where the ultrafiltration membranes are located. The membranes have a surface area of 3.72 m^2 (four membrane modules, each with an area of 0.93 m^2) and a pore size of 0.04 μm (ZW-10, Zenon).

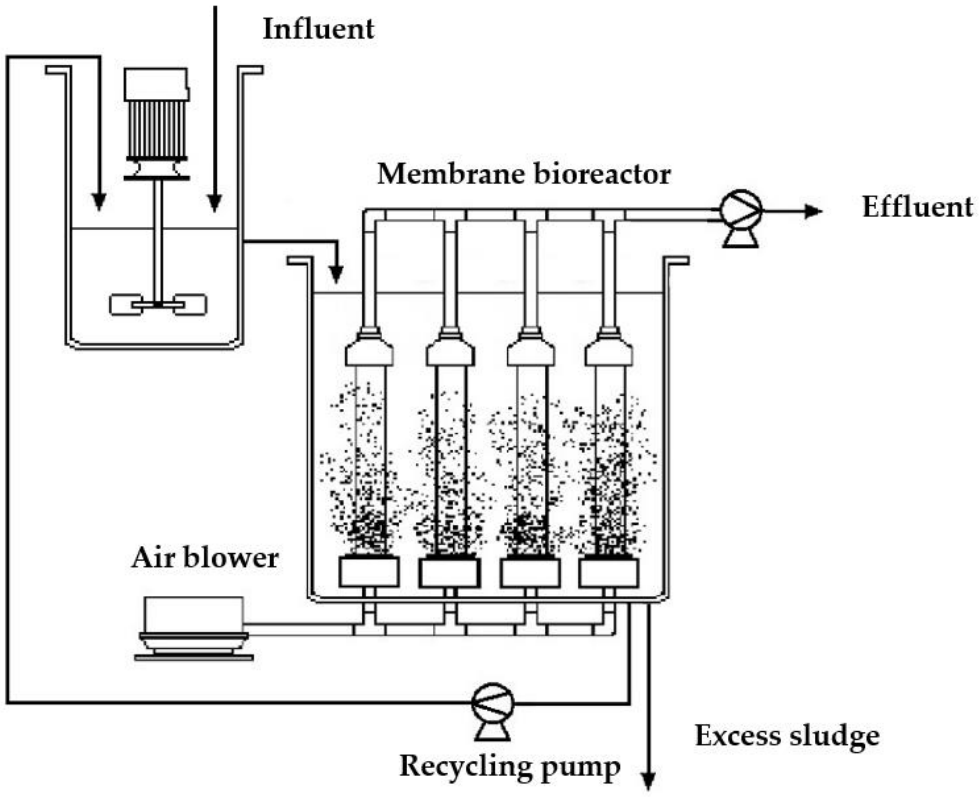


Figure 1. Schematic diagram of the pilot plant of an MBR for municipal wastewater treatment used in the study.

The plant was supplied with urban wastewater from the primary settling chamber of the Los Vados WWTP in Granada. The membrane modules were operated through a peristaltic pump, which cyclically combines a filtration phase spanning 9 min 35 s, followed by a subsequent backwash cycle of 25 s. Filtration is carried out from the outside of the membrane to the inside of the membrane by suction. The biological reactor has a blower that continuously supplies air to clean the membranes. The recirculation from the aerobic membrane tank was to the mixing tank to facilitate mixing of the influent with the recirculation. When the MLSS target is reached, the excess sludge is purged from the system. The study was carried out between May 2020 and December 2021; therefore, the treatment was studied under different seasonal conditions of the influent and temperature.

2.2. Operating Conditions

During the research, four phases of operation of the pilot plant were carried out, in which the MLSS and HRT were modified. The HRT variable and MLSS variable were experimentally selected for comparison and the sludge retention time (SRT) was fixed by the environmental variables. For the start-up, the plant was inoculated with the biological reactor from the Los Vados WWTP. After a period of adaptation of the biomass to the operating conditions, a purge flow rate of the system was set at a stationary state. When working at an HRT of 6 and 12 h, the influent came from the primary settling tank of the Los Vados WWTP. The sludge produced in the primary settling tank was not studied as it belongs to the real plant. The sludge accumulated in the MBR in the pilot plant is a consequence of the influent from the primary settling tank. The plant was in continuous operation, and during the development of all the cycles, samples were taken daily from the influent, mixing tank, bioreactor, and effluent to characterize the wastewater (MLSS, biological oxygen demand in five days (BOD_5), chemical oxygen demand (COD), pH, temperature, and conductivity). Table 1 shows the conditions tested.

Table 1. Operation conditions of the pilot-scale MBR in this study for municipal wastewater treatment. MLSS (mixed liquor suspended solids), HRT (hydraulic retention time), temperature, and SRT (sludge retention time).

Cycle	HRT (h)	MLSS (mg L^{-1})	Average Temperature (°C)	SRT (Day)
1	6	4256 ± 1023	21.4 ± 1.0	22.3
2	6	7477 ± 869	19.1 ± 2.6	10.7
3	12	6151 ± 386	20.0 ± 1.5	38.5
4	12	2888 ± 371	18.0 ± 1.1	36.5

The objective of setting a low HRT in this study is because, previously, insufficient studies have been carried out on how the mixing liquor of an MBR is affected by different pharmaceutical shocks. The MLSS values of the system were set, as they are the usual operating values for a WWTP. This was done by setting a system purge flow rate that kept the MLSS concentration constant, thus imposing an SRT time. The SRTs obtained were high in all cases due to the environmental conditions of the area and its elevated temperatures. The tests were carried out on different days once steady state was reached in each operating cycle.

Once the steady state was reached at the desired conditions, biomass samples were collected for respirometric tests in the laboratory.

2.3. Dosing Study

The compounds selected as the focal pharmaceuticals were ibuprofen, diclofenac, and erythromycin. In addition, the nature of the compounds was also considered in the choice, with only one antibiotic being selected. In particular, erythromycin and diclofenac were on the EU list, and ibuprofen was chosen because of its high detection and widespread use. Nonsteroidal anti-inflammatory pharmaceuticals such as ibuprofen and diclofenac are the most detected pharmaceuticals in water [10,47,48]. Erythromycin removal effectiveness in conventional wastewater treatment plants is 65.6% [49]. The criteria used for the dosing of the sludge were decided for erythromycin and diclofenac based on the value of their solubility in water. Based on this data, three dosing tests were carried out: the first at a concentration 2.5 times lower than their solubility value in water, the second at a concentration equal to the solubility value in water, and the third at a concentration four times higher than the solubility value in water. In the case of ibuprofen, given its high solubility in water, the criterion of using water to establish the different dosing values was meaningless, as research by other authors has shown that the highest value found in wastewater in different areas of the world has been 55.97 µg/L [50]. Therefore, this value was the criterion chosen for the lowest concentration taken (dosing 1). Dosing 2 was at a concentration 2.5 times higher than dosing 1, and dosing 3 was performed at a concentration 10 times higher than dosing 1. Dosing was performed discontinuously in the respirometer. A summary table of the different dosings performed is shown in Table 2.

Table 2. Concentrations of pharmaceutical products for each dosing.

Pharmaceutical	Dosing 1 (mg L^{-1})	Dosing 2 (mg L^{-1})	Dosing 3 (mg L^{-1})
Diclofenac	0.95	2.37	9.48
Erythromycin	0.58	1.44	5.76
Ibuprofen	0.06	0.13	0.56
Mixture	Dosing 1 of the 3 compounds	Dosing 2 of the 3 compounds	Dosing 3 of the 3 compounds

Water solubility (25 °C) of diclofenac: 2.37 mg L^{-1}. Water solubility (25 °C) of erythromycin: 1.44 mg L^{-1}.

2.4. Experimental Procedure

During the steady state of the different studied cycles, the influence of the different pharmaceuticals on the heterotrophic biomass was analyzed in the laboratory. For each test, 1 L of biomass was collected from the membrane bioreactor of the pilot plant. During each cycle, 13 respirometric tests were performed, corresponding to a reference test without dosing and one test for each pharmaceutical dosing, both individually and together. In the laboratory, the sludge was preconditioned to reach endogenous conditions, where any substrate contained in the sample is consumed. Once the sludge was conditioned, the different concentrations of the chosen pharmaceuticals were added.

The sample of 1 L of biomass was introduced into the BM-Advance respirometer for respirometry. The respirometer was operated at a temperature of 20.0 ± 0.1 °C, an air flow rate of 0.906 ± 0.001 L min^{-1}, and a stirring speed of 2000 rpm. In addition to the utilization of mechanical agitation, recirculation from the bottom to the top of the respirometer was facilitated through the operation of a peristaltic pump to promote homogenization of the biomass.

Once the biomass was stable in the respirometer, the dynamic test (constant O_2) was started. Starting from a sodium acetate stock solution with a concentration of 200 mg/L, three sample additions (substrate added 1 (5 mL), substrate added 2 (10 mL), and substrate added 3 (15 mL)) were made to evaluate the evolution of the Rs parameter (dynamic rate of oxygen uptake) over time for the 3 additions of stock solution. The experimental parameters have been designed to guarantee complete substrate consumption, with the highest elimination of substrate being with the peak Rs during the three sequential substrate additions in the dynamic test and with the OUR_{max} in the static test. This observation was maintained in all tests carried out. From the data obtained from the Rs program as a function of time, the kinetic constants were calculated.

Once the breathing test was performed, the static test (without O_2 supply) was carried out. In this test, the evolution of the static oxygen uptake rate (OUR) versus time was obtained. Figure 2 shows an example of the graphs obtained in the different experiments.

From the respirometric test data, the Monod model was employed for the determination of the kinetic parameters that characterize the autotrophic and heterotrophic biomass [51]. The maximum specific growth rate for heterotrophic biomass (μ_m), the half-saturation coefficient for organic matter (K_M), the yield coefficient for heterotrophic biomass (Y_H), and the substrate degradation rate for organic matter removal ($r_{su,\,H}$) were evaluated. The endogenous respiration test was carried out to evaluate the decomposition coefficient for heterotrophic biomass (b_H).

The time and Rs values were obtained for the different tests. Once these data were obtained, the mathematical procedure described by [52] was followed to calculate the oxygen consumption (OC) for the different additions of acetate. All the kinetic constants were then calculated from these data.

2.5. Statistical Analysis

Statistical analysis of the kinetic parameter data of the study was carried out to obtain the regression models. Once the relationship between the input and output variables of the system was obtained, the analysis of variance (ANOVA) tables were generated using Office Statistical Tools of Open Office. The significance of the variables was estimated statistically by calculating the *p*-value with a confidence level of 95%. The regression coefficients were used to obtain contour maps using the response surface methodology approach [53]. The representation of the obtained graphs was made in Python software 3.8.

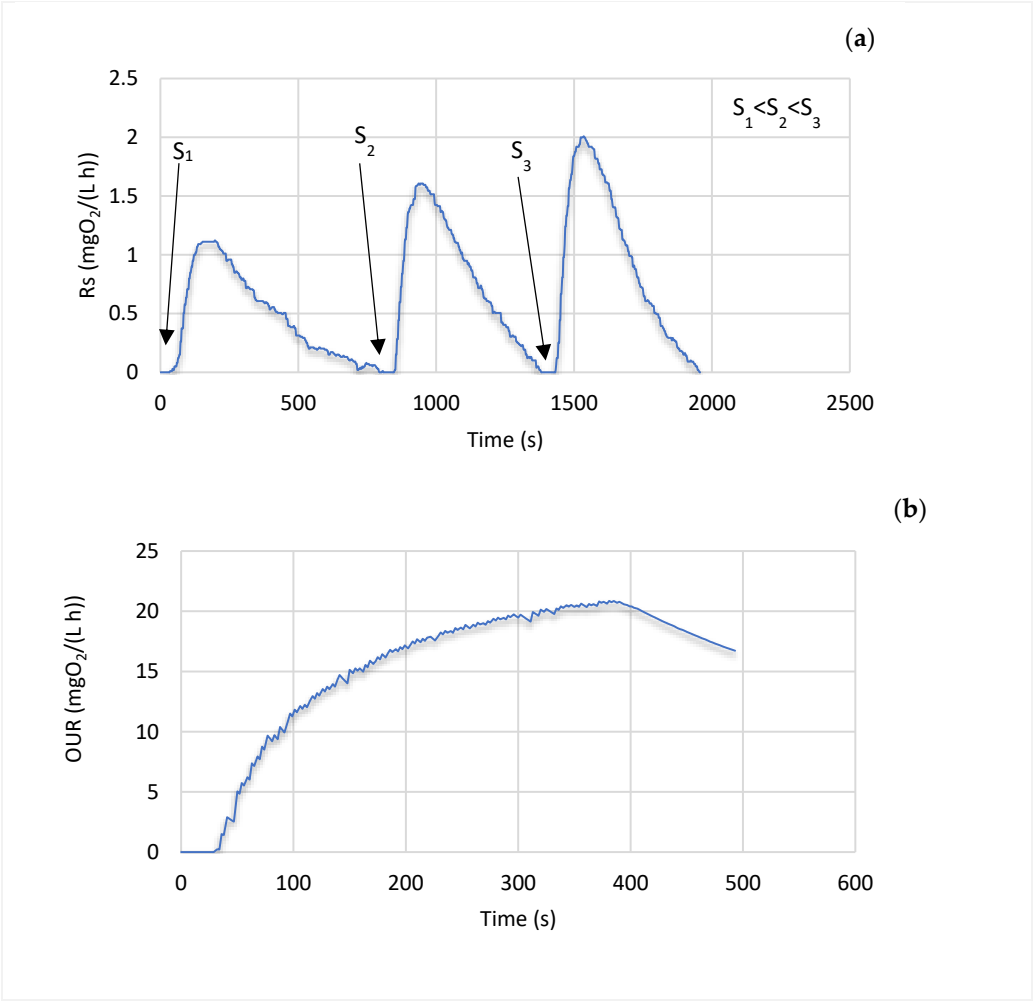


Figure 2. Examples of a graph obtained for the evolution of the dynamic rate of oxygen consumption (Rs) and a graph obtained for the evolution of the static oxygen uptake rate (OUR). (**a**) Dynamic test; (**b**) Static test. S_1: substrate added 1 (5 mL); S_2: substrate added 2 (10 mL); S_3: substrate added 3 (15 mL).

3. Results and Discussion

The influence of fluctuations in the kinetic parameters characterizing heterotrophic biomass in the MBR has been analyzed for each pharmaceutical compound individually and together to identify the cause of possible changes with respect to the reference values (without pharmaceuticals).

3.1. Ibuprofen

Table 3 shows the kinetic parameters for heterotrophic biomass in the absence and presence of ibuprofen at the different operating conditions for each cycle.

The evolution of the $Y_{H,VVS}$ parameter for the pharmaceutical ibuprofen is shown in Figure 3.

The $Y_{H,VVS}$ generally remained constant or slightly higher in the absence of ibuprofen. However, except for cycle 4, in all cycles there is a slight decrease in the first concentration. The μ_m values varied markedly within each cycle of operation without detecting a clear trend in the values themselves. When the reference values were higher than the concentration values, as is the case in cycles 1 and 2 for concentration 1, as well as in cycle 3 for concentrations 1 and 2, this implies that the heterotrophic biomass needs more time to oxidize.

Table 3. Values of the calculated kinetic constants for ibuprofen. $Y_{H,VVS}$ (yield coefficient for heterotrophic biomass), K_M (half-saturation coefficient for organic matter), μ_m (maximum specific growth rate for heterotrophic biomass), b_H (decay coefficient for heterotrophic biomass), r_{su} (substrate degradation rate for organic matter removal), MLSS (mixed liquor suspended solids).

		Average $Y_{H,VVS}$	**K_M**	**μ_m**	**b_H, d^{-1}**	**r_{su} (mg O_2/Lh) (Higher)**	**MLSS (mg L^{-1})**
Cycle 1	Reference	0.59 ± 0.02	3.97	0.009	0.063	12.84	4267
	C1 Ibuprofen	0.57 ± 0.06	2.06	0.004	0.053	9.27	4500
	C2 Ibuprofen	0.59 ± 0.00	8.21	0.016	0.068	15.34	4967
	C3 Ibuprofen	0.58 ± 0.02	11.78	0.018	0.066	12.41	4967
Cycle 2	Reference	0.65 ± 0.00	2.73	0.011	0.067	36.37	7333
	C1 Ibuprofen	0.57 ± 0.04	1.61	0.003	0.101	13.56	6833
	C2 Ibuprofen	0.59 ± 0.01	28.28	0.036	0.078	16.29	7567
	C3 Ibuprofen	0.63 ± 0.01	2.09	0.006	0.095	20.34	7367
Cycle 3	Reference	0.56 ± 0.04	21.42	0.019	0.039	9.14	6200
	C1 Ibuprofen	0.53 ± 0.01	4.19	0.003	0.033	6.91	6867
	C2 Ibuprofen	0.53 ± 0.01	2.74	0.002	0.031	6.74	6867
	C3 Ibuprofen	0.56 ± 0.03	59.73	0.058	0.033	9.17	6133
Cycle 4	Reference	0.64 ± 0.00	2.65	0.013	0.028	15.17	2933
	C1 Ibuprofen	0.66 ± 0.01	4.57	0.021	0.105	18.57	2300
	C2 Ibuprofen	0.65 ± 0.00	16.25	0.073	0.032	18.50	2300
	C3 Ibuprofen	0.65 ± 0.01	6.97	0.019	0.024	16.37	3000

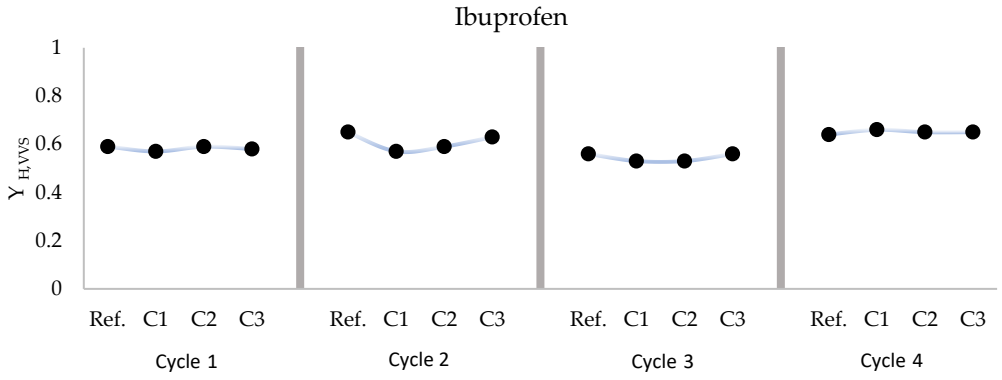


Figure 3. Evolution of $Y_{H,VVS}$ parameter for different concentrations of ibuprofen in the four operation cycles.

Regarding the evolution of the r_{su} parameter, in cycle 1, it decreases for the concentration and then recovers a value close to the reference value for concentrations 2 and 3. In the case of cycle 2, the parameter decreases considerably in the three ibuprofen concentrations with respect to the reference value. This difference in behavior in these two cycles may be due to the different adaptation periods of the biomass to ibuprofen, which is better in cycle 1. Although both cycles have an HRT of 6 h, the biomass of cycle 1 has a much longer SRT (22.3 days) compared to cycle 2 (10.7 days), which makes cycle 1 adapt better, even though it has a lower concentration of MLSS. Other authors have indicated that a higher SRT enhances the diversity of slow-growing bacteria and favors the elimination of compounds such as ibuprofen [54–57], which can favor a good reaction of the biomass to dosing. Furthermore, this behavior could also be favored by the temperature (21.4 °C and 19.1 °C for cycles 1 and 2, respectively), which, being higher in cycle 1, may cause

the micro-organisms to have a higher metabolic activity and be able to better withstand the effect of the toxicant in the system. This behavior occurs in a similar way for cycles 3 and 4, but with a less pronounced variation, being that the adaptation of the heterotrophic biomass to ibuprofen is slightly more favorable in cycle 3. In this case, due to the similarity of the SRT parameters (cycle 3, 38.5 days; cycle 4, 36.5 days) and the fact that both operate at a HRT of 12 h, the average temperature of cycle 3 is 20 °C, which is higher than that of cycle 4 (18 °C), which makes the biomass of cycle 3 adapt more quickly to the different ibuprofen concentrations. These variations of the parameter r_{su} also occur for the parameter b_H, where in the case of cycle 4 there is a very sharp increase in the concentration 1 of pharmaceutical compound that may be caused by a chemical stress of the system due to the addition of ibuprofen. This behavior has been reported by other authors, who note that when the ibuprofen concentration was increased, the microbial activity was affected [58]. Furthermore, the inhibitory effect of ibuprofen was confirmed by another author [59].

3.2. Diclofenac

Table 4 shows the kinetic parameters for heterotrophic biomass in the absence and presence of diclofenac at the different operating conditions for each cycle.

Table 4. Values of the calculated kinetic constants for diclofenac. $Y_{H,VVS}$ (yield coefficient for heterotrophic biomass), K_M (half-saturation coefficient for organic matter), μ_m (maximum specific growth rate for heterotrophic biomass), b_H (decay coefficient for heterotrophic biomass), r_{su} (substrate degradation rate for organic matter removal), MLSS (mixed liquor suspended solids).

		Average $Y_{H,VVS}$	**K_M**	**μ_m**	**bH, d^{-1}**	**r_{su} (mg O_2/Lh) (Higher)**	**MLSS (mg L^{-1})**
Cycle 1	Reference	0.55 ± 0.03	2.64	0.005	0.029	7.92	3133
	C1 Diclofenac	0.55 ± 0.05	4.46	0.006	0.022	6.96	3133
	C2 Diclofenac	0.50 ± 0.04	1.62	0.003	0.027	6.98	3367
	C3 Diclofenac	0.57 ± 0.02	1.59	0.004	0.023	8.83	3367
Cycle 2	Reference	0.65 ± 0.00	2.73	0.011	0.067	36.37	7333
	C1 Diclofenac	0.65 ± 0.01	4.04	0.016	0.047	36.62	7333
	C2 Diclofenac	0.64 ± 0.01	1.35	0.009	0.076	29.95	5800
	C3 Diclofenac	0.62 ± 0.00	16.09	0.021	0.046	13.60	5967
Cycle 3	Reference	0.56 ± 0.04	21.42	0.019	0.039	9.14	6200
	C1 Diclofenac	0.61 ± 0.03	3.9	0.004	0.026	13.66	6000
	C2 Diclofenac	0.62 ± 0.00	2.90	0.005	0.027	11.58	6000
	C3 Diclofenac	0.58 ± 0.03	2.00	0.003	0.047	9.87	5667
Cycle 4	Reference	0.64 ± 0.00	2.65	0.013	0.028	15.17	2933
	C1 Diclofenac	0.65 ± 0.00	ND	ND	0.037	15.57	2617
	C2 Diclofenac	0.65 ± 0.00	2.74	0.015	0.033	16.79	2617
	C3 Diclofenac	0.66 ± 0.00	2.50	0.017	0.035	19.84	2617

ND: non detected.

The evolution of the $Y_{H,VVS}$ parameter for the pharmaceutical diclofenac is shown in Figure 4.

In the case of diclofenac, the $Y_{H,VVS}$ remained constant during the first concentration but decreased during the second concentration, and there was an increase above the reference value for the third concentration. For the constant μ_m, in the case of cycles 1 and 3, there was a noticeable decrease in its value, implying that as the amount of pharmaceutical added to the activated sludge increases, it decreases in activity and needs more time to oxidize. However, this does not occur in the case of cycles 2 and 4, where its value remains

constant or increases. This is because the shock produced by the diclofenac causes the micro-organisms to increase their activity to counteract the toxicant.

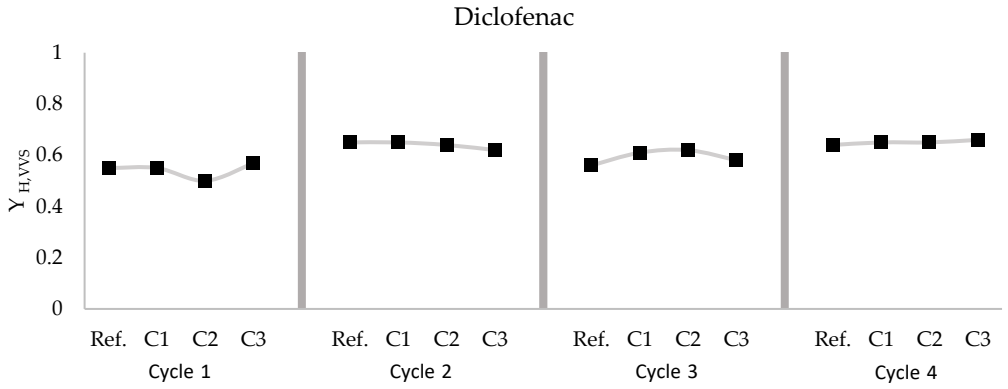


Figure 4. Evolution of $Y_{H,VVS}$ parameter for different concentrations of diclofenac in the four operation cycles.

In the case of the r_{su} parameter, for cycle 1 it remains approximately constant; however, a considerable drop is observed in cycle 2 as the diclofenac concentration increases. This indicates that the micro-organisms have been affected and a decay of the system has occurred. This behavior is similar to that which occurred in the case of ibuprofen; the system behaves the same against the anti-inflammatory substances tested, as the system is favored by a higher temperature in cycle 1, which seems to make them more metabolically active and better able to withstand the toxin introduced against a higher concentration of MLSS. However, the biomass does not show the same behavior for cycles 3 and 4 and does not seem to have a clear trend, which seems to be due to a higher retention time, which makes the biomass more stabilized and react better to a toxicant. This is the same behavior as the other anti-inflammatory substance studied (ibuprofen), which is in line with the findings of other authors [54–57,60] that pointed out that a higher SRT increases bacterial diversity and favors the reaction to compounds such as diclofenac.

3.3. Erythromycin

Table 5 shows the kinetic parameters for heterotrophic biomass in the absence and presence of erythromycin at the different operating conditions for each cycle.

The evolution of the $Y_{H,VVS}$ parameter for the pharmaceutical erythromycin is shown in Figure 5.

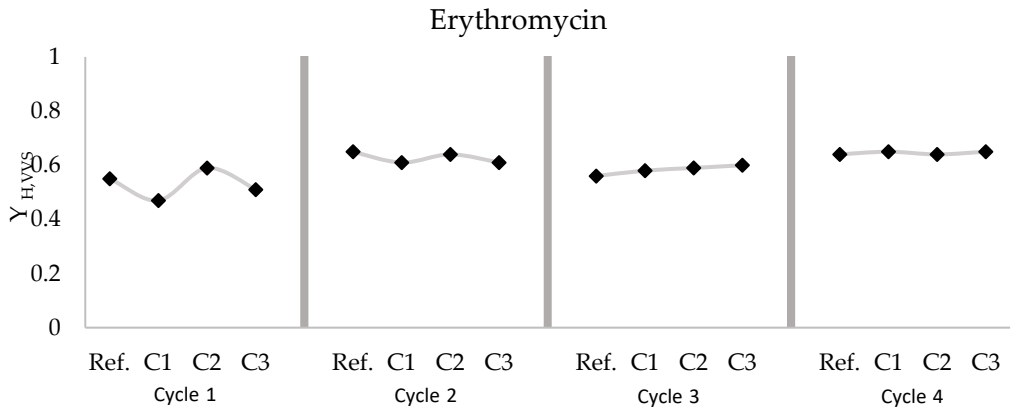


Figure 5. Evolution of $Y_{H,VVS}$ parameter for different concentrations of erythromycin in the four operation cycles.

Table 5. Values of the calculated kinetic constants for erythromycin. $Y_{H,VVS}$ (yield coefficient for heterotrophic biomass), K_M (half-saturation coefficient for organic matter), μ_m (maximum specific growth rate for heterotrophic biomass), b_H (decay coefficient for heterotrophic biomass), r_{su} (substrate degradation rate for organic matter removal), MLSS (mixed liquor suspended solids).

		Average $Y_{H,VVS}$	K_M	μ_m	b_H (d^{-1})	r_{su} (mg O_2 L^{-1} h^{-1}) (Higher)	MLSS (mg L^{-1})
Cycle 1	Reference	0.55 ± 0.03	2.64	0.005	0.029	7.92	3133
	C1 Erythromycin	0.47 ± 0.03	5.50	0.004	0.033	5.71	3800
	C2 Erythromycin	0.59 ± 0.01	3.26	0.005	0.042	7.92	3800
	C3 Erythromycin	0.51 ± 0.04	2.05	0.003	0.031	8.52	3833
Cycle 2	Reference	0.65 ± 0.00	2.73	0.011	0.067	36.37	7333
	C1 Erythromycin	0.61 ± 0.03	3.33	0.006	0.053	19.18	8033
	C2 Erythromycin	0.64 ± 0.01	1.91	0.006	0.026	23.80	8233
	C3 Erythromycin	0.61 ± 0.02	4.40	0.008	0.022	18.82	8233
Cycle 3	Reference	0.56 ± 0.04	21.42	0.019	0.039	9.14	6200
	C1 Erythromycin	0.58 ± 0.02	22.98	0.026	0.034	10.34	6133
	C2 Erythromycin	0.59 ± 0.02	5.46	0.008	0.035	10.92	6000
	C3 Erythromycin	0.60 ± 0.01	2.22	0.004	0.030	11.91	6000
Cycle 4	Reference	0.64 ± 0.00	2.65	0.013	0.028	15.17	2933
	C1 Erythromycin	0.65 ± 0.01	19.45	0.068	0.024	18.34	3000
	C2 Erythromycin	0.64 ± 0.00	3.73	0.016	0.017	18.54	3183
	C3 Erythromycin	0.65 ± 0.01	1.71	0.009	0.016	13.12	3183

The $Y_{H,VVS}$, independent of the temperature, MLSS, and HRT during the first concentration of the pharmaceutical, decreases compared to an increase for concentration 2, which resulted in a higher biomass activity. This seems to indicate that the micro-organisms, faced with the antibiotic effect, increase their activity to counteract the harmful effect produced. In the case of μm, the values remain constant in the case of cycle 1, indicating that the microbial activity does not seem to have been affected. However, in the case of cycle 2, it decreases markedly, indicating that the microorganisms have lost the oxidative capacity, being affected by the antibiotic. This also occurs in the case of cycle 3, where their activity is greatly reduced, with the exception of concentration 1. The same occurs in the case of cycle 4, where their oxidation capacity is reduced. As there is a similar effect in all cycles, it appears that the main variable affecting the system is the antibiotic nature of the compound, with milder effects being seen with respect to the HRT and MLSS concentrations. Other authors obtained the same effect on biomass, obtaining a negative impact on the microbial growth by reducing the maximum heterotrophic growth rate [61]. Another study reported a similar behavior in anaerobically treated biomass, where at low concentrations, erythromycin does not affect the biomass because it has a slight resistance, but it is affected at higher concentrations where it negatively affects the biomass because it is an antibiotic [62].

3.4. Mixture of Ibuprofen, Diclofenac, and Erythromycin

Table 6 shows the kinetic parameters for heterotrophic biomass in the absence and presence of a mixture of ibuprofen, diclofenac, and erythromycin at the different operating conditions for each cycle.

Table 6. Values of the calculated kinetic constants for a mixture of ibuprofen, diclofenac, and erythromycin. $Y_{H,VVS}$ (yield coefficient for heterotrophic biomass), K_M (half-saturation coefficient for organic matter), μ_m (maximum specific growth rate for heterotrophic biomass), b_H (decay coefficient for heterotrophic biomass), r_{su} (substrate degradation rate for organic matter removal), MLSS (mixed liquor suspended solids).

		Average $Y_{H,VVS}$	K_M	μ_m	bH, d^{-1}	r_{su} (mg O_2/Lh) (Higher)	MLSS (mg L^{-1})
Cycle 1	Reference	0.59 ± 0.02	3.97	0.009	0.063	12.84	4267
	C1 Mixture	0.58 ± 0.01	5.97	0.009	0.064	13.37	4267
	C2 Mixture	0.56 ± 0.02	9.98	0.007	0.035	11.64	6100
	C3 Mixture	0.60 ± 0.01	ND	ND	0.030	15.35	6100
Cycle 2	Reference	0.65 ± 0.00	2.73	0.011	0.067	36.37	7333
	C1 Mixture	0.63 ± 0.00	3.65	0.007	0.023	21.97	8800
	C2 Mixture	0.60 ± 0.01	2.07	0.004	0.013	17.89	7933
	C3 Mixture	0.58 ± 0.01	8.86	0.009	0.013	14.62	7767
Cycle 3	Reference	0.56 ± 0.04	21.42	0.019	0.039	9.14	6200
	C1 Mixture	0.56 ± 0.02	7.94	0.009	0.040	10.09	5733
	C2 Mixture	0.59 ± 0.04	20.89	0.027	0.034	13.58	5733
	C3 Mixture	0.54 ± 0.06	52.44	0.031	0.023	7.53	6633
Cycle 4	Reference	0.64 ± 0.00	2.65	0.013	0.028	15.17	2933
	C1 Mixture	0.63 ± 0.00	2.37	0.012	0.033	15.66	3000
	C2 Mixture	0.65 ± 0.00	1.73	0.010	0.013	13.11	3367
	C3 Mixture	0.63 ± 0.00	13.99	0.054	0.024	17.41	3432

ND: non detected.

The evolution of the $Y_{H,VVS}$ parameter for the mixture of pharmaceuticals is shown in Figure 6.

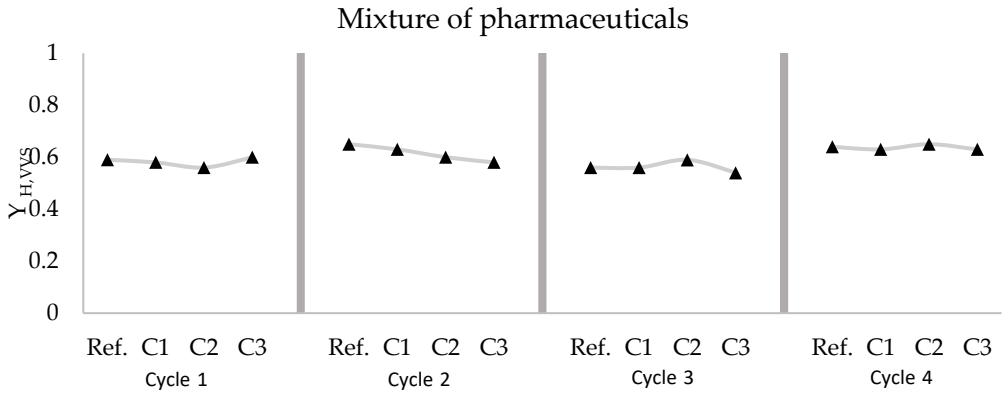


Figure 6. Evolution of $Y_{H,VVS}$ parameter for the different concentrations of the mixture of pharmaceuticals in the four operation cycles.

In cycle 2, when the pharmaceutical mixture was added to the system for all three concentrations, the heterotrophic biomass exhibited a decrease in the rate of organic matter degradation, which is in line with a decrease in the b_H parameter, indicating that less heterotrophic biomass was oxidized as the pharmaceutical concentration increased. Therefore, it is observed that the um parameter decreases for increasing concentrations in this cycle, as well as $Y_{H,VVS}$, indicating that the system needs more time to oxidize.

However, the behavior of the system observed in cycle 2 does not occur in the other cycles, which are not as affected by the addition of the pharmaceuticals because they are able to buffer the toxic shock better. This may be because the SRT of these cycles is much higher than that of cycle 2, which means that the biomass is more stable and is not affected as much by the variations that are being introduced. Figure 7 shows the evolution of r_{su} parameters in all cases.

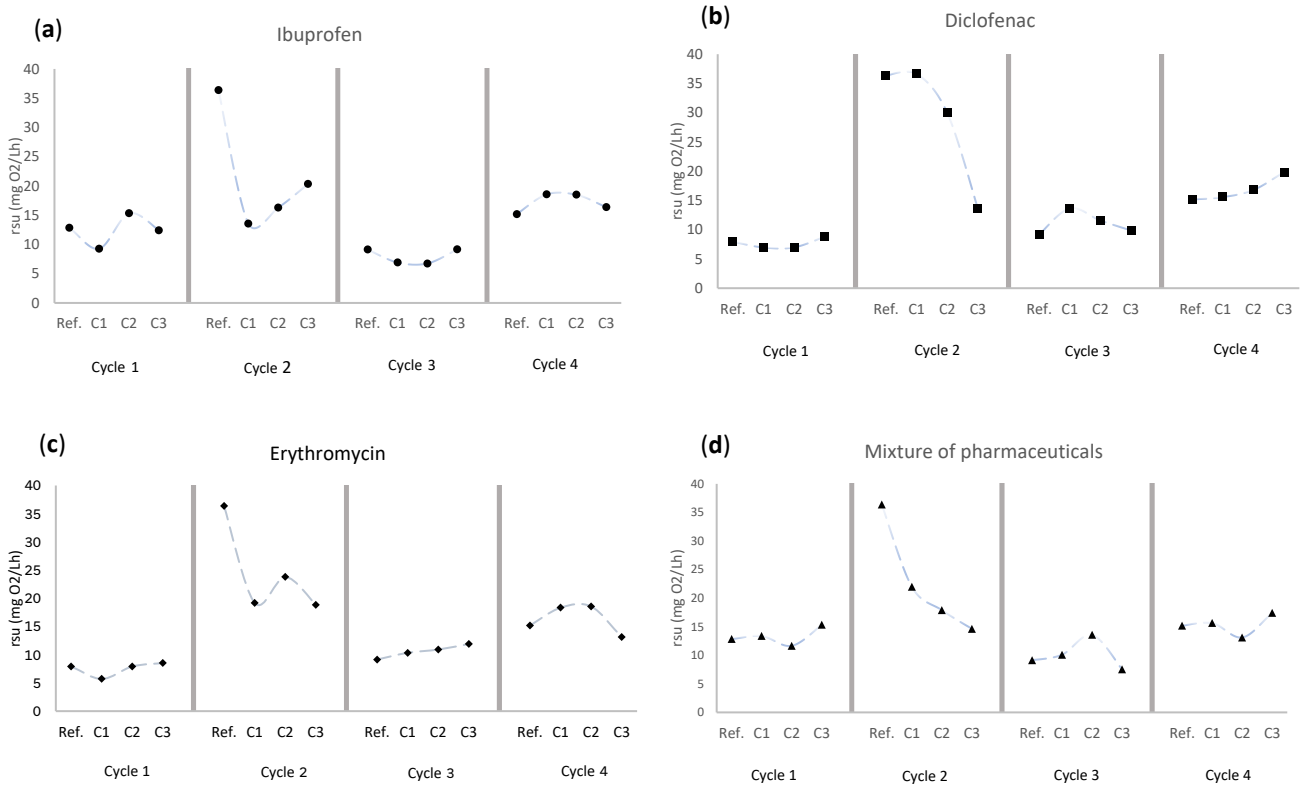


Figure 7. Evolution of the r_{su} parameter (Ref. (reference concentration), C1 (concentration 1), C2 (concentration 2), C3 (concentration 3). (**a**) Evolution of the r_{su} (mg O_2/L h) for ibuprofen in different cycles. (**b**) Evolution of the r_{su} (mg O_2/L h) for diclofenac in different cycles. (**c**) Evolution of the r_{su} (mg O_2/L h) for erythromycin in different cycles. (**d**) Evolution of the r_{su} (mg O_2/L h) for the mixture of pharmaceuticals (ibuprofen, diclofenac, and erythromycin) in different cycles.

Based on the r_{su} data obtained and in order to evaluate their evolution, an analytical statistical study was carried out.

3.5. Combined Effect of Operational Variables

An analytical statistical study was carried out to observe the influence of different system variables on the r_{su} and r_x (heterotrophic biomass growth rate) parameters. Different response surfaces were obtained. The variables analyzed were the effect of the concentration of the pharmaceutical compounds (mg L^{-1}), the variation of the volatile solids in suspension, and the HRT.

The adjusted general equation obtained in the study for the r_{su} and r_x variables is shown in Equation (1):

$$-a + b{\cdot}HRT + c{\cdot}X + d{\cdot}[pharmaceutical] - e{\cdot}HRT{\cdot}X + f{\cdot}HRT{\cdot}[pharmaceutical] - g{\cdot}X{\cdot}[pharmaceutical] - h{\cdot}[pharmaceutical]^2 \quad (1)$$

The corresponding values for r_{su} for the different pharmaceuticals are presented in Table 7.

Table 7. Values of the variables obtained for general equation for r_{su}.

	a	b	c	d	e	f	g	h
Ibuprofen	322.142	73.6801	0.204046	−704.479	0.0222387	273.9939	−0.130788	172.215
Diclofenac	1343.2	144.411	0.50484	14.9641	0.0435368	3.62735	0.00723289	1.79009
Erythromycin	974.425	109.527	0.331094	7.85758	0.0276471	1.76038	0.00155738	5.36507
Mixture of pharmaceuticals	182.835	58.7241	0.177247	28.9007	0.0188858	1.77263	0.00168898	5.36507

The corresponding values for r_x for the different pharmaceuticals are presented in Table 8.

Table 8. Values of the variables obtained for general equation for r_x.

	a	b	c	d	e	f	g	h
Ibuprofen	236.385	51.3016	0.123095	−393.148	0.0143061	12.2028	−0.0846876	109.116
Diclofenac	949.434	106.258	0.356699	26.2874	0.0324628	2.64388	0.0053223	2.34626
Erythromycin	768.074	82.166	0.254193	0.0943917	0.0215321	2.28943	0.0033023	3.35068
Mixture of pharmaceuticals	206.625	46.3584	0.126267	21.27	0.0138022	1.29993	0.000956014	0.57605

In the case of ibuprofen, the variable X corresponds to the MLSS (mg/L). For diclofenac, erythromycin, and the mixture of pharmaceuticals, the variable X corresponds to the VSS (mg/L). Tables 9 and 10 show the correlation coefficient values for each pharmaceutical, as well as the optimal values of the variables.

Table 9. Correlation coefficient values and optimal values for r_{su}.

	R^2	HRT Optimal (h)	Optimal (Maximum) Value		
			r_{su} (mg O_2 L^{-1} h^{-1})	VSS (mg L^{-1})	[Pharmaceutical] (mg L^{-1})
Ibuprofen	0.9922	6	690.44	6433	0.56
Diclofenac	0.9597	6	1090.46	6433	Not significant
Erythromycin	0.8739	6	847.32	7033	0.68
Mixture of pharmaceuticals	0.9491	6	669.195	7933	0.238

Table 10. Correlation coefficient values and optimal values for r_x.

	R^2	HRT Optimal (h)	Optimal (Maximum) Value		
			r_x (mg VSS L^{-1} h^{-1})	VSS (mg L^{-1})	[Pharmaceutical] (mg L^{-1})
Ibuprofen	0.9819	6	402.52	6433	0.56
Diclofenac	0.9332	6	729.673	6433	0.0032
Erythromycin	0.9200	6	603.40	7033	Not significant
Mixture of pharmaceuticals	0.9545	6	413.593	7033	Not significant

Analysis of variance (ANOVA) and the associated probability (*p*-value) were obtained for the different variables considered. A confidence level of 95% was established, so that a *p*-value higher than 0.05 was not considered statistically significant on the output variable

(r_{su} and r_x in our case). The significant variables that most affect the system are the HRT and the MLSS. Table 11 shows the *p*-values obtained in the analysis of variance.

Table 11. *p*-Values of the analysis of variance.

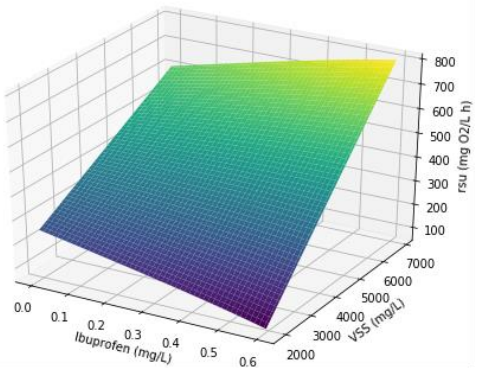
Variable	Ibuprofen		Diclofenac		Erythromycin		Mixture of Pharmaceuticals	
	r_{su} *p*-Value	r_x *p*-Value	r_{su} *p*-Value	r_x *p*-Value	r_{su} *p*-Value	r_x *p*-Value	r_{su} *p*-Value	r_x *p*-Value
HRT	0.0006 *	0.0266 *	0.0081 *	0.0138 *	0.2809	0.1310	0.0173 *	0.0255 *
MLSS	0.0001 *	0.0041 *	0.0016 *	0.0248 *	0.0046 *	0.0024 *	0.5519	0.3989
[Pharmaceutical]	0.8611	0.7099	0.8709	0.5363	0.3490	0.1272	0.0172 *	0.0133 *
HRT · MLSS	0.0000 *	0.0000 *	0.0001 *	0.0002 *	0.0021 *	0.0005 *	0.0005 *	0.0002 *
HRT · [pharmaceutical]	0.0072 *	0.1263	0.0782	0.1405	0.7240	0.4120	0.1341	0.0904
MLSS · [pharmaceutical]	0.0001 *	0.0011*	0.0916	0.1560	0.8492	0.4777	0.2860	0.3330
[pharmaceutical]^2	0.4488	0.6195	0.5953	0.4503	0.6824	0.6395	0.2637	0.2364

* Significant values.

Based on the obtained *p*-value results, the HRT and MLSS were the parameters exhibiting the highest statistical significance across various operational cycles. Specifically, the HRT assumes significant values, except for its relevance in the case of erythromycin. The MLSS was of notable significance, except in the case of the pharmaceutical mixture. The joint consideration of both HRT and MLSS consistently maintains statistical significance. However, when these variables are analyzed in combination with the pharmaceutical concentrations under investigation, they no longer retain statistical significance.

From the regression coefficients obtained, contour maps were obtained using the response surface methodology approach. The response surface plots obtained for the studied variables can be seen in Figure 8.

(**a**)



(**b**)

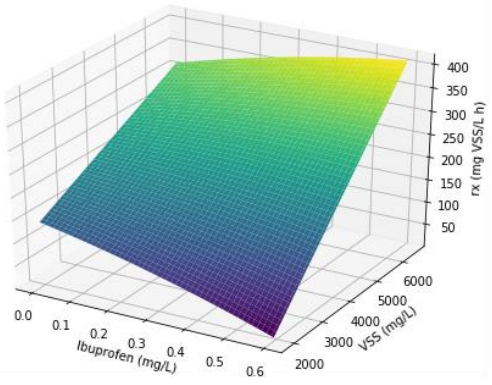


Figure 8. *Cont.*

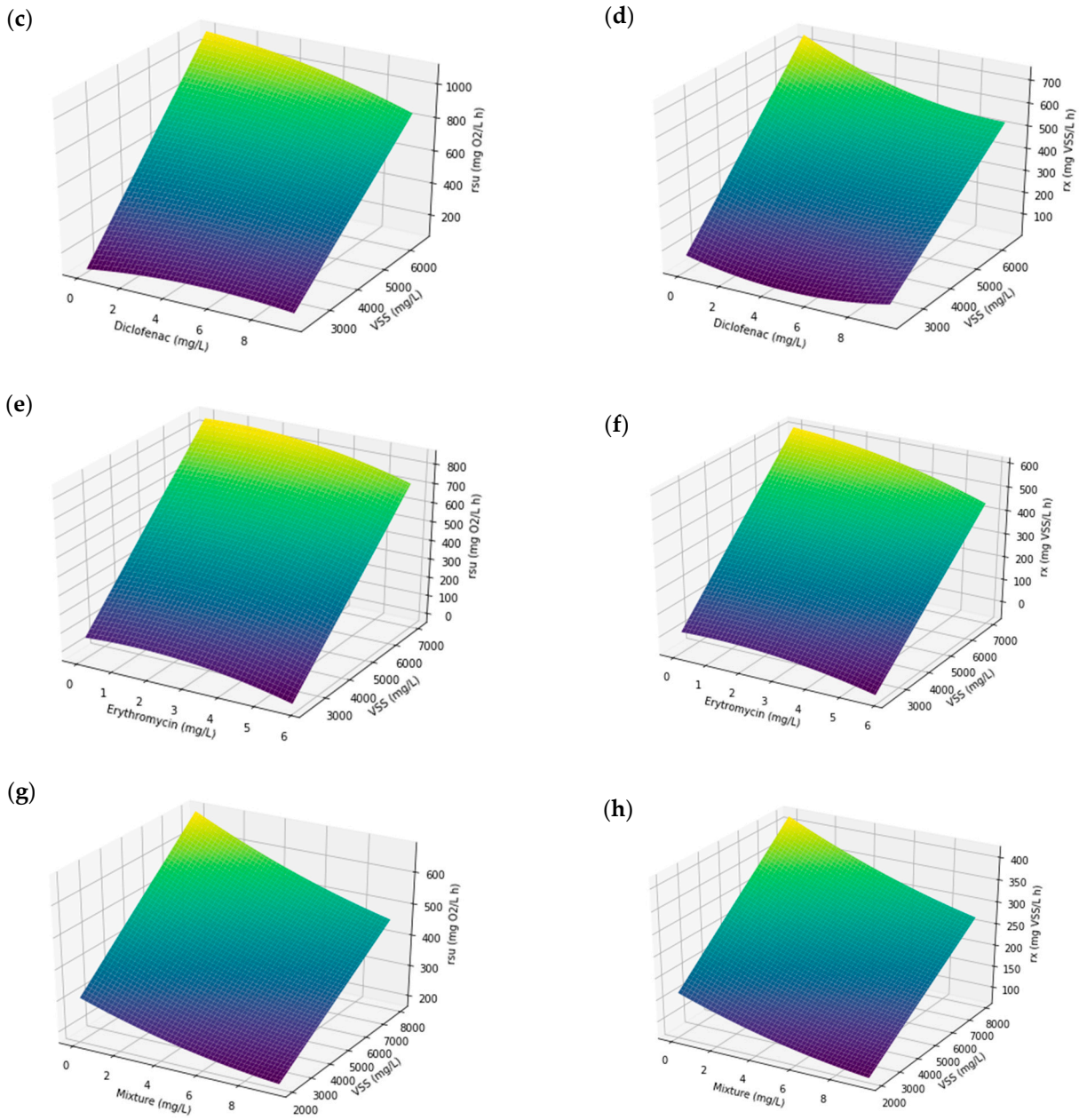


Figure 8. Response surface: (**a**) Response surface for r_{su} (mg O_2/L h) for ibuprofen; (**b**) Response surface for r_x (mg VSS/L h) for ibuprofen; (**c**) Response surface for r_{su} (mg O_2/L h) for diclofenac; (**d**) Response surface for r_x (mg VSS/L h) for diclofenac; (**e**) Response surface for r_{su} (mg O_2/L h) for erythromycin; (**f**) Response surface for r_x (mg VSS/L h) for erythromycin; (**g**) Response surface for r_{su} (mg O_2/L h) for mixture of pharmaceuticals (ibuprofen, diclofenac, and erythromycin); (**h**) Response surface for r_x (mg VSS/L h) for mixture of pharmaceuticals (ibuprofen, diclofenac, and erythromycin).

The type of pharmaceutical compound affects the biomass in one way or another, with the erythromycin having the greatest effect on the system. This may be due to the nature of the compound itself, as it is an antibiotic. In general terms, for the biomass analyzed, the optimal (most active) HRT is 6 h, as expected, because when the substrate is constant, the biomass characteristics are better. Therefore, if the HRT is fixed, it is observed that the greater quantity of microorganisms related to the MLSSVs results in the greater response of the system. Other authors have reported that the MBR is not able to improve their performance by increasing the HRT and MLSS against a pharmaceutical such as diclofenac [63]. Another study based on the biomass of a rotating annular bioreactor reported

that erythromycin changes the microbial structure by selecting for resistant bacteria, but at low concentrations it does not affect the biological process [64], in agreement with the statistical study in this article.

The response variables r_{su} and r_x have a time lag between them. The substrate effect occurs before the biomass effect, which is expected, because the substrate drop is detected first in the system and the optimum biomass growth occurs later. In the case of the pharmaceutical compound mixture, the effect of the variables is lower than that of the individual pharmaceutical compounds when they are analyzed individually, so it is deduced that there is a synergic effect between the pharmaceutical compounds themselves. In practical terms, these results allow us to predict the behavior of the biomass against pharmaceuticals discharges at different concentrations.

4. Conclusions

This study analyzed the dosing of three ascending concentrations of pharmaceutical compounds individually, along with their combined effects, on the heterotrophic biomass of a MBR. The pilot plant operated with real urban wastewater under operating conditions of two HRT, different concentrations of MLSS, and different SRT. Based on the kinetic results acquired, the following conclusions were obtained:

- At a HRT of 6 h, the heterotrophic biomass showed a higher microbial activity than a HRT of 12 h and the effect of the pharmaceutical on the biomass is higher. Regardless of the MLSS concentration and pharmaceutical type, the higher SRT causes the lower effect of dosing in the heterotrophic biomass. Furthermore, the erythromycin is the most affected pharmaceutical in the heterotrophic biomass since it is an antibiotic.
- The higher temperature at a HRT of 6 h had less of an effect on the behavior of heterotrophic biomass under the presence of pharmaceuticals.
- Different response surfaces of the system were obtained to predict the expected behavior of the biomass against possible spills of the pharmaceuticals studied. When the biomass is dosed with the pharmaceuticals individually, a greater kinetic response is produced than when it is doped with a combination of the three pharmaceuticals. This slower kinetic response in the mixture of diclofenac, ibuprofen, and erythromycin indicates that there is a synergistic effect between them.

Considering the above, the system favorably absorbed the effect of the pharmaceutical compounds and showed a better response at lower HRT. Therefore, the MBR presents itself as a promising technology for urban wastewater treatment against emerging contaminants such as diclofenac, ibuprofen, and erythromycin, and has the ability to proactively predict a course of action in response to potential discharge.

Author Contributions: Conceptualization, J.M.P.C., M.d.M.M.M. and J.M.P.; methodology, J.M.P.C. and M.d.M.M.M.; validation, J.M.P. and M.d.M.M.M.; formal analysis, L.A.B., V.D.M., J.M.P.C., M.d.M.M.M. and J.M.P.; investigation, L.A.B., V.D.M., J.M.P.C., M.d.M.M.M. and J.M.P.; data curation, L.A.B. and V.D.M.; writing—original draft preparation, L.A.B. and V.D.M.; writing—review and editing, J.M.P.C., M.d.M.M.M. and J.M.P.; visualization, L.A.B. and V.D.M.; supervision, J.M.P.C., M.d.M.M.M. and J.M.P. All authors have read and agreed to the published version of the manuscript.

Funding: Project PID2021-124740NB-I00 financed by MCIN/AEI/10.13039/501100011033/ and by FEDER A way of doing Europe.

Data Availability Statement: Data are contained within article.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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