Biological absorption as main route for amoxicillin reduction and heterotrophic kinetic modeling in a "NIPHO" bioreactor

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ABSTRACT

This research analyzes the different amoxicillin (AMX) removal routes, i.e. biodegradation, biological absorption and adsorption, within a "NIPHO" activated sludge reactor treating urban wastewater. Moreover, the impact of different AMX concentrations (15-75 mg L^{-1}) on the kinetic performance of the heterotrophic biomass was evaluated. The "NIPHO" bioreactor worked at 4.8±0.7 h of hydraulic retention time, 20.9±1.4ºC of temperature, and 2000±120 mgTSS L^{-1} of mixed liquor total suspended solids. High Pressure Liquid Chromatography was used to evaluate AMX removal by the different pathways and a respirometric method was applied to model the heterotrophic kinetics in absence and presence of AMX. Additionally, a multivariable statistical analysis was carried out to determine the influence of the operation variables on the response of the system. The results showed that the removal of AMX was essentially carried out via biological absorption (11.06-87.13%), and biomass concentration and sludge volume index were the most influential variables on it. Additionally, the study concluded that the heterotrophic biomass of the bioreactor was

not significantly influenced by AMX, although the net heterotrophic biomass growth rate at 75 mg L^{-1} AMX concentration showed statistically significant differences with the rest of concentrations, and was reduced up to 8.84 mgVSS L^{-1} h^{-1}.

Keywords: Absorption; Amoxicillin; Heterotrophic biomass; Kinetic modeling; Removal route; Respirometry

Abbreviations: **AMX**, amoxicillin; **BOD_5**, five-day biochemical oxygen demand; **COD**, chemical oxygen demand; **DO**, dissolved oxygen; **HRT**, hydraulic retention time; **MLSS**, mixed liquor suspended solids; **OC**, oxygen consumption; **OUR**, static oxygen uptake rate; **SRT**, sludge retention time; **SVI**, sludge volume index; **T**, temperature; **TN**, total nitrogen; **TSS**, total suspended solids; **VSS**, volatile suspended solids; **WWTP**, wastewater treatment plant.

1. Introduction

During last thirty years, the presence of new organic pollutants considered as persistent but scarcely legislated, called ‘emerging pollutants’, has increased in wastewater and aquatic environment [1]. Among the different categories in which these micropollutants are divided, pharmaceuticals are one of the most significant groups detected [2]. Specifically, antibiotics are highly used for human therapy, veterinary medicine, agriculture and aquaculture [3, 4]. In fact, some authors, like Gelband et al. [5], indicated an increase (higher than 30%) in the global consumption of antibiotics in the first decade of the century, (from 50 to 70 billion standard units), however, others like Wise [6] or Baghapour et al. [7] did not report a rise in the antibiotic world consumption although indicated a global use between 100 and 200 millions of kilograms in 2002 and 2012, respectively. Furthermore, Versportent et al. [8] reported Spain as one of the European Union countries with a higher consumption of antibiotics. Besides, its global extensive use has resulted in an increase of its presence in different water bodies [7] since a high percentage of antibiotics, which are consumed by humans and animals, are excreted without being metabolized or in conjugated forms of the initial pharmaceutical. This implies their transfer to the aquatic environment through rain or wastewater [9], when the unused antibiotics are disposed to the environment as occurs in pharmaceutical industry, hospitals and residential facilities waste [4]. Moreover, a global concern has appeared for the last years since these substances and their degradation products are persistent, which leads to its bioaccumulation in the environment [3, 10-11]. In this regard, apart from the immune toxicity, neurotoxicity, endocrine disruption and carcinogenicity consequences of the direct exposure to these contaminants [12], the main problems may result in the human consumption of food

where the pollutants have been bioaccumulated leading to undesired results in human health like immune-allergic responses or the development, transfer and spread of antibiotic-resistant bacteria, which compromise the effectiveness of pharmaceuticals as the antibiotic resistance of microorganisms is becoming more and more problematic [4, 13].

Among the most important antibiotic groups in medicinal and veterinary therapy, penicillins containing β-lactam antibiotics are remarkable because they are widely consumed. In fact, they are responsible of the 50-70% of the global antibiotic consumption [3]. Amoxicillin (AMX), which belongs to this category, was also included in the last Commission Implementing Decision (EU) 2018/840 published by the European Union [14]. It is a semi-synthetic drug which is effective against a wide range of infections caused by gram-positive and gram-negative bacteria and is used for the treatment and prevention of respiratory, gastrointestinal, urinary and skin bacterial infections [4]. Furthermore, since above 75% of amoxicillin consumed is not metabolized and it is released to wastewater [15], several research have been conducted in order to study the effect of its presence in the aquatic environment and its removal pathways [4, 16-18]. Among the most common biological removal pathways, i.e. assimilation or biological absorption, biosorption or biological adsorption and biodegradation [19], these last two of them have been reported as the main ones for the reduction of drugs [20-21]. However, investigation about the most significant AMX removal routes has not been widely investigated yet [22].

Additionally, although biological treatments implemented in the wastewater treatment plants (WWTPs) have been reported not to guarantee an efficient removal of antibiotics [23-27], there is scarce information about the impact of these micropollutants on the

activated sludge within the bioreactor. Several authors have studied the effect of some pharmaceutical emerging pollutants on the heterotrophic and/or autotrophic biomass of an activated sludge reactor [26, 28-29]. However, there are no studies related to the impact of AMX on the heterotrophic kinetic of this kind of biological reactor, although Rezaei et al. [22] studied two common models to predict the removal rate of substrate in a membrane bioreactor-hollow fiber (MBR-HF).

Thus, the main aim of this research was to evaluate the AMX removal pathways in a variation of the conventional activated sludge (CAS) reactor, called NIPHO reactor, of the WWTP of Villapérez (Asturias, Spain). This study was complemented by the simulation and assessment of the effect of an intrusion of AMX on the heterotrophic bacteria through its kinetic modeling by a respirometric technique.

2. Materials and methods

2.1. NIPHO bioreactor and sampling procedure

The WWTP of Villapérez (Asturias, Spain), which has a NIPHO biological reactor, was studied in this research. This WWTP treats wastewater from the Central University Hospital of Asturias and urban wastewater from Oviedo, Siero, Noreña, Llanera and Sariego councils. The pretreatment includes a coarse pit, two bar screens for coarse and fine solids, a grit removal and a degreasing area prior to the primary settling and the NIPHO biological process.

NIPHO configuration is based on an activated sludge system combining anoxic, anaerobic and aerobic zones, as shown in Fig. 1.

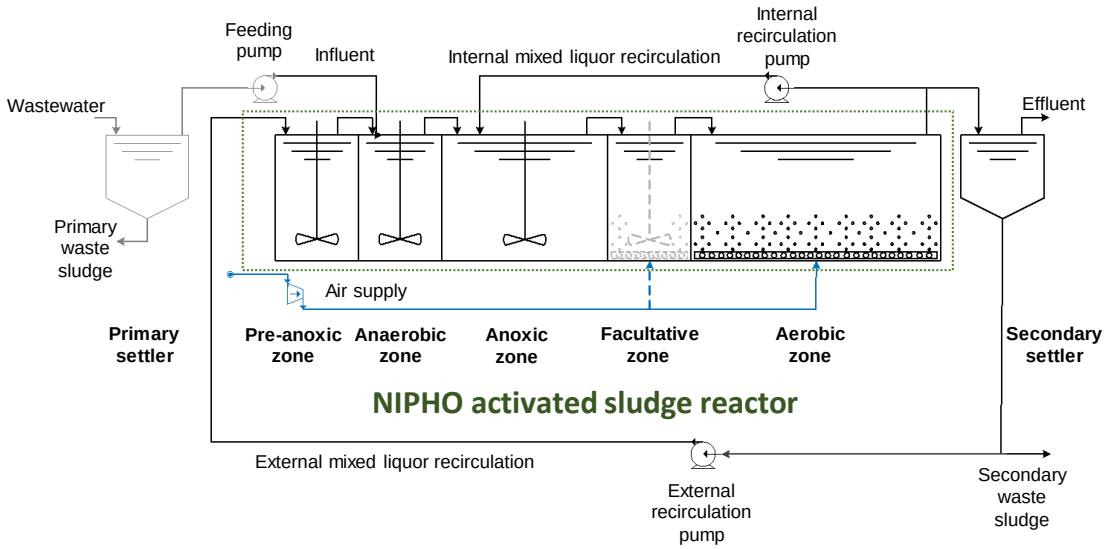

Fig. 1. Description of the NIPHO activated sludge reactor of the WWTP of Villapérez (Asturias, Spain).

The research period extended from June to October 2019 (five months) in order to collect activated sludge samples corresponding to the steady state of the NIPHO bioreactor. The values of the operation variables are indicated in Table S1.

The determination of chemical oxygen demand (COD), five-day biochemical oxygen demand (BOD_5), total suspended solids (TSS), volatile suspended solids (VSS), sludge volume index (SVI), total phosphorus (TP) and PO_4-P concentration were carried out according to APHA [30]. Total nitrogen (TN) and NH_4-N concentration were assessed by using a cuvette kit for nitrogen determination provided by Merck. This test is in accordance with the UNE-EN-ISO 11905-1.

A 2 L sample of activated sludge was, twice a week, specifically collected from the aerobic zone of the NIPHO biological reactor the day before the respirometric test. These samples had a mixed liquor total suspended solids (X_{TSS}) of 2000±120 mgTSS L^{-1}. Afterwards, sludge samples were aerated for 18 h to ensure the total removal of the

substrates contained in the mixed liquor (endogenous conditions) before starting the respirometric tests [31] and the evaluation of the AMX removal routes.

2.2. Evaluation of the biological removal pathways

Activated sludge samples were taken to analyze the AMX either assimilated, adsorbed or biodegraded by the activated sludge once finished the respirometric tests. Firstly, the reactor liquor was put in a 1 L test tube in order to settle until a clear separation between the supernatant and the sludge volume was observed (at least 30 min of settling). The sedimented sludge was washed by distilled water until a final volume of 300 mL, to remove the AMX dissolved in the remaining supernatant, and to desorb the AMX from the sludge. Then, the washed sludge was sedimented by a second time and sonicated for 62 min [32] by a SONOREX RK 100 ultrasonic bath supplied by Bandelin (Equipment Nominal Power:80 W, Frequency: 35kHz, V:230V, I:0.4 A). The sonication was carried out to break the sludge floccules and to release the AMX absorbed by the biomass. A 2 mL sample of washed and sonicated sludge and 2 mL of the supernatant were filtered by a syringe nylon filters supplied by Dismed S.A. (pore size: 0.45 µm) and analyzed by High Pressure Liquid Chromatography (HPLC).

AMX determination was carried out through Agilent 1200 with an UV-VIS detector operating in a wavelength of 230 nm [15] and a 150 mm ZORBAX Extend-C18 column (Agilent, Santa Clara, CA 95051, USA). Solvents used for High Pressure Liquid Chromatography (HPLC) were ultrapure water (Milli-Q) (solvent A) and acetonitrile (≥99.9%, CAS No.: 75-05-8, MW: 41.05 g mol^{-1}) (solvent B), which was supplied by VWR Chemicals (Fontenay-sous-Bois, F-94126, France). Elution of AMX started with 5% solvent B for 10 min linear gradient to 53% solvent B with a flow rate of 1 mL min^{-}

[1]. The operation temperature was 30ºC and the sample volume of injection was 40 µL [15].

Add to AMX, due to its fast hydrolysis to various degradation products, piperazine-2,5-dione oligomer and AMX dimer were detected in the HPLC [3, 33]. The quantification of AMX was carried out by considering both the degradation products and the number of carbon atoms, according to the procedure proposed in the literature. In this regard, Eq. (1) enables to calculate the real concentration of AMX (A_{AMX}):

$$A_{AMX} = RF_{Pip} \cdot A_{Pip} + 2 \cdot RF_{Dim} \cdot A_{Dim} + A_{AMX,det} \qquad (1)$$

where A_{Pip} is the area of the AMX piperazine-2,5-dione oligomer, A_{Dim} is the area of the AMX dimer, $A_{AMX,det}$ is the area of AMX detected in the HPLC, RF_{Pip} is the response factor for piperazine-2,5-dione (1.1) and RF_{Dim} is the response factor for dimer (0.64) [34].

2.3. Assessment of the heterotrophic kinetics

2.3.1. Respirometric assays

The respirometric test began with the introduction of the preconditioned active sludge in a 1 L biological batch reactor. This bioreactor was provided with a jacket connected to a water thermostatic bath to keep the temperature of the activated sludge samples at 20.0±0.1ºC. A 1 L min^{-1} air flow rate, measured by a rotameter, was continuously supplied to the activated sludge by a diffuser fed by an air pump. The homogeneity in the mixed liquor was accomplished by a mechanical stirrer, which operated at 150 rpm. Additionally, the pH had a constant value of 7.44±0.23 in the period of study (Table S1). Dissolved oxygen (DO) and temperature were continuously measured in the mixed

liquor sample by the optical O_2 electrode LDO70 (oximeter XS, OXY70) and a built-in temperature sensor, respectively. A scheme of the experimental setup used for the respirometric assays is shown in Fig. S1.

Each experiment day, two respirometric tests were done in absence and presence of AMX at different concentrations of study (15, 30, 45, 60 and 75 mg L^{-1}) in order to determine the heterotrophic kinetic parameters in absence and presence of the antibiotic. The tests were performed with high concentrations in order to assess the effect of AMX on the heterotrophic kinetics at most unfavorable conditions; additionally, this range of concentration allowed for monitoring the changes in AMX concentration and carrying out fractionation for the evaluation of the biological removal pathways. Each of the respirometric tests consisted of an initial exogenous part and a final endogenous part performed in the presence and absence of a continuous supply of air, respectively [31]. In presence of AMX, once a constant DO line was ensured, the exogenous part started by adding the required volume of the 2000 mg L^{-1} AMX stock solution to obtain the desired concentration in the mixed liquor samples. Once the DO trend line was stable, the experiment continued with the 10 mL sodium acetate additions at increasing concentrations, which were prepared from a 500 mg L^{-1} stock solution (250, 400 and 500 mg L^{-1}). As a consequence of the degradation of the substrate by the heterotrophic bacteria, a decrease in the DO of the mixed liquor was observed after each addition. The endogenous test was carried out without any additional substance and the absence of air was ensured. In absence of AMX, the experiment was carried out in a similar way without AMX addition.

2.3.2. Estimation of heterotrophic kinetic parameters

The dynamic oxygen uptake rate (R_S) and the static oxygen uptake rate (OUR) were calculated through the derivation of DO depending on the time for the exogenous and endogenous respirometric experiments, respectively (Fig. S2 and Fig. S3, respectively). In this way, heterotrophic kinetic parameters such as the decay coefficient in absence and presence of AMX ($b_{H,n/AMX}$ and $b_{H,AMX}$, respectively), the yield coefficient in absence and presence of AMX ($Y_{H,n/AMX}$ and $Y_{H,AMX}$, respectively), the maximum specific growth rate in absence and presence of AMX ($\mu_{m,H,n/AMX}$ and $\mu_{m,H,AMX}$, respectively) and the half-saturation coefficient for carbon source in absence and presence of AMX ($K_{M,n/AMX}$ and $K_{M,AMX}$, respectively), were obtained from the endogenous part of the respiration test (the first of them) and from the exogenous part of the respirometric assay (the rest of them), according to the procedure proposed by Leyva-Díaz et al. [28]. These two last kinetic parameters ($\mu_{m,H}$ and K_M) were calculated by applying the linearization of the Monod model equation, which assumes that the biomass growth rate occurs due to substrate limitation [35]. The supplementary parameters required for the heterotrophic kinetic study are specified in Text S1 in the Supplementary Material.

The analysis of heterotroph kinetics was complemented by the evaluation of the degradation rate for carbon source in absence and presence of AMX ($r_{su,H,n/AMX}$ and $r_{su,H,AMX}$, respectively) and the net heterotrophic biomass growth rate in absence and presence of AMX ($r'_{x,H,n/AMX}$ and $r'_{x,H,AMX}$, respectively) [28].

2.4. Statistical analysis

A multivariable statistical analysis was addressed by Canoco 4.5 for Windows (ScientiaPro, Budapest, Hungary). This software allowed for determining the influence of the operation variables on the removal rate for AMX biodegradation ($RR_{AMX,biod}$), removal rate for AMX absorption ($RR_{AMX,abs}$), removal rate for AMX adsorption ($RR_{AMX,ads}$), heterotrophic kinetic parameters, $r_{su,H}$ and $r'_{x,H}$. Additionally, SPSS 22.0 was used to evaluate the existence of statistically significant differences between the results obtained for the $r_{su,H}$ and $r'_{x,H}$ in absence and presence of AMX. The software conditions for both statistical programs are indicated in Text S2 in the Supplementary Material.

3. Results

3.1. Amoxicillin (AMX) removal routes

A mass balance is proposed in Eq. (2) in order to determine the contributions of the different AMX removal routes. In this regard, the initial amount of AMX ($m_{AMX,i}$) is the sum of the following contributions: biodegraded, not discarding other chemical routes ($m_{AMX,biod}$), dissolved in the supernatant at the end of the respirometric test ($m_{AMX,dis}$), absorbed in the sludge ($m_{AMX,abs}$), and, finally, adsorbed on the sludge ($m_{AMX,ads}$).

$$m_{AMX,i} = m_{AMX,biod} + m_{AMX,abs} + m_{AMX,ads} + m_{AMX,dis} \qquad (2)$$

In that way, both $m_{AMX,i}$ and $m_{AMX,dis}$ are obtained from the liquor HPLC analysis. The mass of biodegraded AMX was obtained from the ratio of consumed oxygen and chemical oxygen demand (COD) due to AMX, being the oxygen consumption determined from the numerical integration of R_S after the addition of each AMX

concentration in the respirometric test (Fig. S4). The mass of absorbed AMX was determined from the concentration of the washed sonicated sludge, and, finally, the adsorbed AMX mass is obtained by difference.

Fig. 2 shows the AMX removal rates via biodegradation, absorption and adsorption.

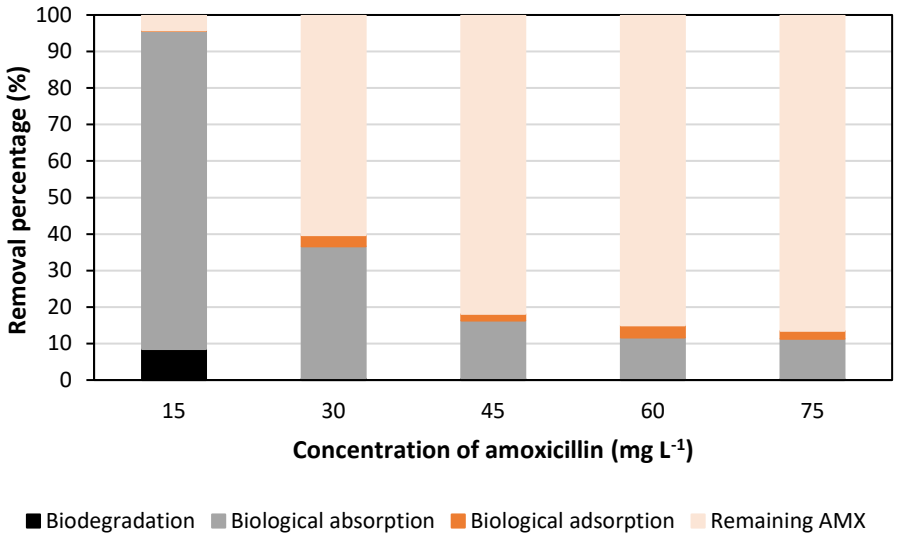

Fig. 2. Evaluation of the amoxicillin (AMX) removal pathways for AMX concentrations of 15, 30, 45, 60 and 75 mg L^{-1}.

AMX was biodegraded at removal rates varying from 0.21 to 8.47% for the AMX concentrations tested in this research (15-75 mg L^{-1}). The highest removal percentage (8.47%) corresponded to the lowest AMX concentration (15 mg L^{-1}), which was supported by the highest values of R_S for the addition of AMX (Fig. S4a) with a maximum value close to 3.5 mgO_2 L^{-1} h^{-1}. However, the rest of additions of higher AMX concentrations caused a reduction of the maximum values of R_S, which were, in general, below 2.5 mgO_2 L^{-1} h^{-1} (Fig. S4b-e). Regarding the contribution of the biological adsorption to the removal of AMX, the removal rate ranged from 0.19 to 3.36% (Fig. 2). It should be noted that biological absorption is the major process of

removal of AMX in the NIPHO activated sludge reactor, with $RR_{AMX,abs}$ varying from 11.06 to 87.13% (Fig. 2).

Fig. 3a shows the results of the multivariable statistical analysis of the different AMX removal pathways depending on the operation variables.

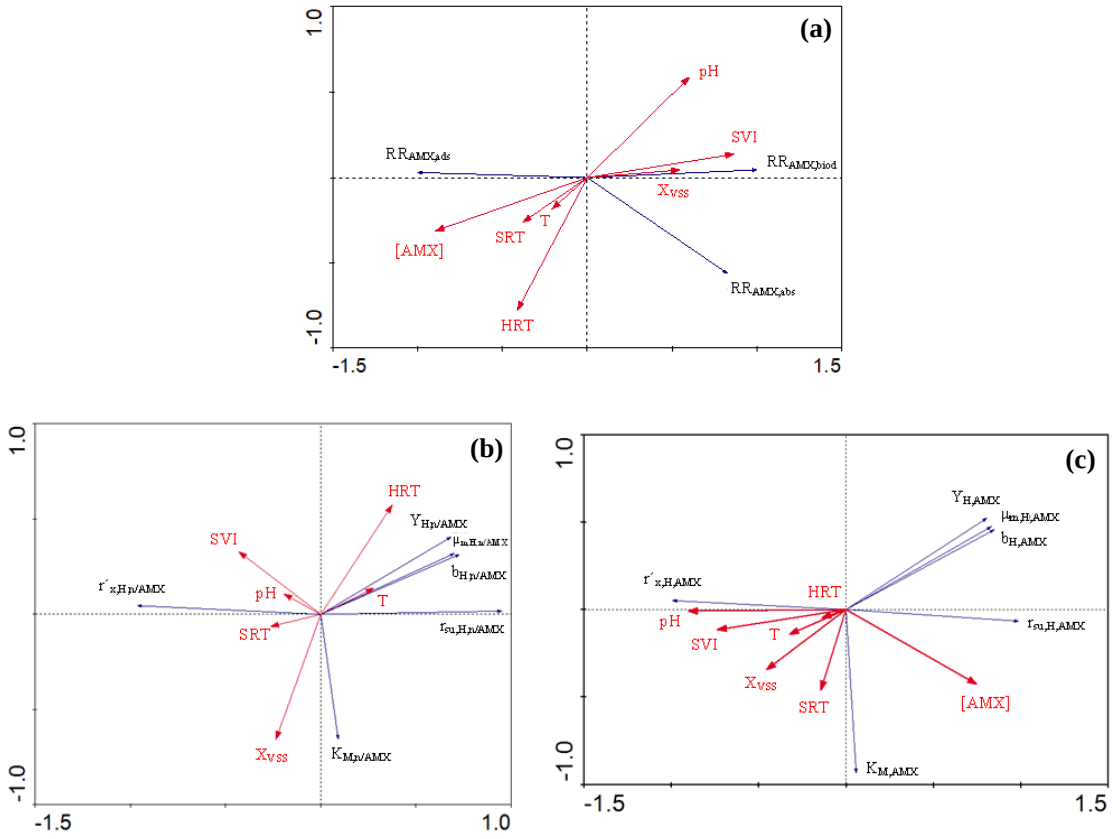

Fig. 3. Triplot diagram for redundancy analysis of the removal rate for AMX biodegradation ($RR_{AMX,biod}$), removal rate for AMX absorption ($RR_{AMX,abs}$) and removal rate for AMX adsorption ($RR_{AMX,ads}$) in relation to the operation variables HRT, X_{VSS}, T, SRT, SVI, pH and concentration of AMX (a), and triplot diagram for redundancy analysis of the heterotrophic kinetic parameters (Y_H, $\mu_{m,H}$, K_M and b_H), degradation rate for carbon source ($r_{su,H}$) and net heterotrophic biomass growth rate ($r'_{x,H}$) in relation to the same operation variables in absence of amoxicillin (AMX) (b) and presence of AMX (c). [AMX] represents the concentration of AMX.

It should be highlighted that the removal rates for AMX absorption and biodegradation ($RR_{AMX,abs}$ and $RR_{AMX,biod}$) had a positive correlation with two operation variables closely related to biomass, such as mixed liquor volatile suspended solids (X_{VSS}) and sludge volume index (SVI). However, the most detrimental operation variable was the concentration of AMX, which was negatively correlated with $RR_{AMX,abs}$ and $RR_{AMX,biod}$. In relation to $RR_{AMX,ads}$, it had a positive correlation with operation variables closely related to residence time, such as sludge retention time (SRT) and hydraulic retention time (HRT), as well as temperature and concentration of AMX. Additionally, $RR_{AMX,ads}$ was negatively correlated with pH.

3.2. Heterotrophic kinetic modeling

The results of respirometric test times and maximum oxygen uptake rates from Fig. S2 and S3 are developed in Text S3 in the Supplementary Material. The heterotrophic kinetic parameters were assessed from the different evolutions for R_S and OUR through the respirometric procedure indicated in section 2.3.2. Table 1 shows the kinetic parameters for heterotrophic bacteria in absence and presence of AMX for the different concentrations evaluated.

The values of Y_H were higher in presence of AMX ($Y_{H,AMX}$) for concentrations of 15, 45 and 60 mg L^{-1}, with increases from 0.8 to 6.2%, producing a higher amount of heterotrophic biomass per carbonaceous substrate oxidized in presence of AMX. The contrary trend is observed for 30 and 75 mg L^{-1} AMX concentrations, with reductions of 1.0 and 2.6%, respectively, and a lower amount of heterotrophs produced per carbon source biodegraded. Furthermore, the values of $Y_{H,AMX}$ were reduced when the AMX

concentration increased in relation to the lowest concentration (15 mg L^{-1}), as shown in Table 1.

Table 1. Kinetic parameters for modeling the heterotrophic biomass in absence and presence of amoxicillin (AMX).

Parameter	Concentration of amoxicillin (mg L^{-1})				
	15	**30**	**45**	**60**	**75**
	Absence of AMX				
$Y_{H,n/AMX}$ (mgVSS mgCOD^{-1})	0.5860±0.0030	0.5698±0.0059	0.5719±0.0447	0.5843±0.0161	0.5959±0.0357
$\mu_{m,H,n/AMX}$ (h^{-1})	0.0105±0.0043	0.0178±0.0022	0.0169±0.0014	0.0145±0.0049	0.0159±0.0006
$K_{M,n/AMX}$ (mg O_2 L^{-1})	0.1612±0.0424	3.7603±0.8383	2.7207±0.3448	2.0451±1.3322	2.0930±1.3602
$b_{H,n/AMX}$ (day^{-1})	0.0784±0.0163	0.0800±0.0042	0.0878±0.0103	0.1005±0.0094	0.1541±0.0406
	Presence of AMX				
$Y_{H,AMX}$ (mgVSS mgCOD^{-1})	0.6248±0.0162	0.5642±0.0173	0.5765±0.0179	0.6011±0.0184	0.5806±0.0299
$\mu_{m,H,AMX}$ (h^{-1})	0.0130±0.0102	0.0157±0.0011	0.0171±0.0033	0.0180±0.0029	0.0126±0.0010
$K_{M,AMX}$ (mgO_2 L^{-1})	0.4780±0.1166	3.1602±0.4096	2.4443±1.0861	2.7419±0.7305	1.2254±0.4484
$b_{H,AMX}$ (day^{-1})	0.0652±0.0318	0.0620±0.0196	0.0988±0.0217	0.1019±0.0053	0.1279±0.0156

A similar trend was observed for $\mu_{m,H}$ with increase percentages varying from 1.2 to 19.4%, with the exception of 30 and 75 mg L^{-1} AMX concentrations. In these two last concentrations, the reduction rates were 11.8 and 20.8% for 30 and 75 mg L^{-1}, respectively, implying more time to oxidize carbonaceous substrate by heterotrophs in presence of AMX. It should be highlighted that the lowest value of $\mu_{m,H}$ (0.0126 h^{-1}) corresponded to the highest AMX concentration (75 mg L^{-1}).

Regarding the values of b_H, they decreased in presence of AMX, except for the concentration of 45 mg L^{-1}. The highest AMX concentration (75 mg L^{-1}) had the greatest value of b_H (0.1279 day^{-1}). As indicated in Table 1, the quantity of heterotrophic biomass oxidized per day ranged from 6.20 to 12.79% in presence of AMX.

Fig. 4 shows the values of $r_{su,H}$ and $r'_{x,H}$ in absence and presence of AMX for the different concentrations analyzed of this emerging pollutant.

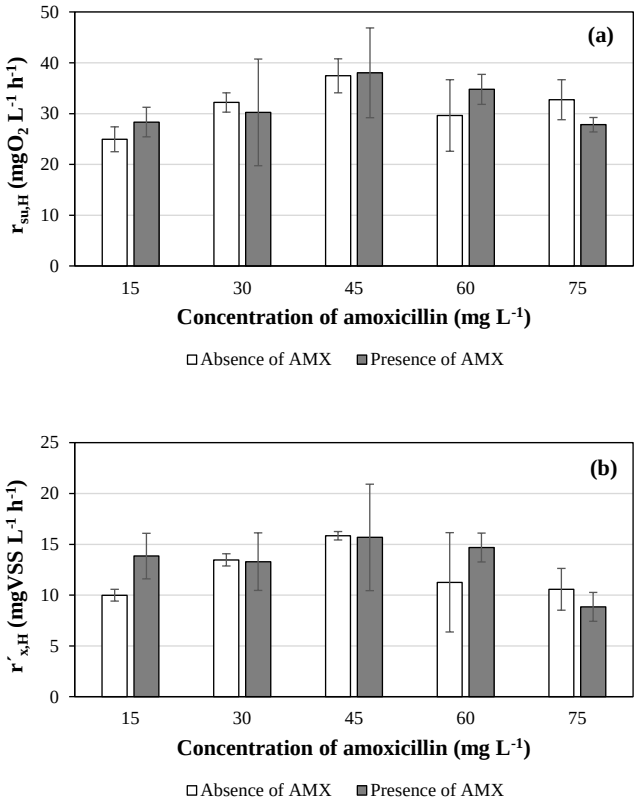

Fig. 4. Degradation rate for carbon source ($r_{su,H}$) (a), and net heterotrophic biomass growth rate ($r'_{x,H}$) (b) in absence and presence of amoxicillin (AMX).

In Fig. 4a it is observed a slightly increasing trend of $r_{su,H}$ in presence of AMX for concentrations ranging from 15 to 60 mg L^{-1}. Despite this, there were no statistically significant differences between $r_{su,H}$ in absence and presence of AMX for the different

concentrations tested since the p-values obtained from the LSD post hoc procedure surpassed α=0.05, according to Table 2 including p-values of ANOVA analysis for the comparison between $r_{su,H}$ and $r'_{x,H}$ in absence and presence of AMX. However, $r_{su,H,AMX}$ showed statistically significant differences between 15 and 45 mg L^{-1} with a p-value of 0.0349, and between 45 and 75 mg L^{-1} with a p-value of 0.0175 (Table 2). A similar behavior resulted from the comparison between $r'_{x,H}$ in absence and presence of different AMX concentrations, without statistically significant differences (p-values > 0.05) (Table 2 and Fig. 4b). It should be noted that there were statistically significant differences between $r'_{x,H,AMX}$ corresponding to 75 mg L^{-1} and the rest of concentrations, with p-values of 0.0482, 0.0478, 0.0110 and 0.0143 for 15, 30, 45 and 60 mg L^{-1}, respectively (Table 2). In general, the comparison between $r_{su,H,n/AMX}$ or $r'_{x,H,n/AMX}$ for the blanks of different AMX concentrations did not show statistical significant differences (Table 2).

Regarding the variables that most influenced the heterotrophic kinetics, Fig. 3b-c show the results of the multivariable statistical analysis in absence and presence of AMX. In absence of AMX, $Y_{H,n/AMX}$, $\mu_{m,H,n/AMX}$ and $b_{H,n/AMX}$ had a positive correlation with HRT and temperature, and a negative correlation with SRT and X_{VSS} (Fig. 3b). In relation to $K_{M,n/AMX}$, it was directly proportional to X_{VSS}. However, it was negatively correlated with pH, SVI and HRT. Moreover, $r_{su,H,n/AMX}$ had a strongly positive correlation with temperature, and $r'_{x,H,n/AMX}$ was positively correlated with SRT, pH and SVI.

Table 2. p-values of sequential comparison (ANOVA analysis) of the degradation rate for carbon source ($r_{su,H}$) and net heterotrophic biomass growth rate ($r'_{x,H}$) in absence and presence of amoxicillin.

		Degradation rate for carbon source ($r_{su,H}$)				
		Blank of amoxicillin concentration(1) / Concentration of amoxicillin(2) (mg L^{-1})				
		15	**30**	**45**	**60**	**75**
Blank of amoxicillin concentration(1) / Concentration of amoxicillin(2) (mg L^{-1})	**15**	-	0.6504	0.0349	0.1105	0.8948
	30	0.1019	-	0.0821	0.2514	0.5313
	45	0.0093	0.2276	-	0.4069	0.0175
	60	0.2372	0.5058	0.0579	-	0.0590
	75	0.0590	0.8927	0.2335	0.3746	-
		Comparison between $r_{su,H,n/AMX}$ and $r_{su,H,AMX}$				
		0.4282	0.6445	0.8913	0.1499	0.1694
		Net heterotrophic biomass growth rate ($r'_{x,H}$)				
Blank of amoxicillin concentration(1) / Concentration of amoxicillin(2) (mg L^{-1})	**15**	-	0.8298	0.4854	0.7265	0.0482
	30	0.1956	-	0.3653	0.5602	0.0478
	45	0.0383	0.3695	-	0.6751	0.0110
	60	0.5956	0.3602	0.0700	-	0.0143
	75	0.8043	0.2366	0.0409	0.7501	-
		Comparison between $r'_{x,H,n/AMX}$ and $r'_{x,H,AMX}$				
		0.1542	0.9438	0.9503	0.1239	0.4189

(1) The results of the statistical comparison of $r_{su,H}$ and $r'_{x,H}$ in absence of amoxicillin ($r_{su,H,n/AMX}$ and $r'_{x,H,n/AMX}$) are shown in the white grids.
(2) The results of the statistical comparison of $r_{su,H}$ and $r'_{x,H}$ in presence of amoxicillin ($r_{su,H,AMX}$ and $r'_{x,H,AMX}$) are shown in the gray grids.

The presence of AMX modified the results obtained in absence of AMX, as observed in Fig. 3c. In this case, the concentration of AMX was the variable that most influenced $r_{su,H,AMX}$, $Y_{H,AMX}$, $\mu_{m,H,AMX}$ and $b_{H,AMX}$, whereas the rest of variables were negatively correlated with these species. Regarding $K_{M,AMX}$, it had a strongly positive correlation with SRT. In general, $r'_{x,H,AMX}$ was positively correlated with all the operation variables and had a negative correlation with the concentration of AMX.

4. Discussion

The reduction of $RR_{AMX,biod}$ for AMX concentrations higher than 15 mg L^{-1} (0.21-0.36%) could be explained by the higher chance of exposure and penetration into the cells, which are essential for biodegradation, at higher concentration gradients of this antibiotic [7]. In this regard, Alexy et al. [36] did not detect any significant biodegradation of AMX in a Closed Bottle Test, with $RR_{AMX,biod}$ of 3% and 5% after 14 and 28 days, respectively. Githinji et al. [15] also concluded that AMX was probably not biodegraded efficiently in WWTPs.

The values obtained for $RR_{AMX,ads}$ could be explained by the low value for the solid-water partition coefficient (K_d) of AMX in activated sludge, which is 1.06 L $kgMLSS^{-1}$ according to Jones et al. [37]. Abegglen et al. [38] indicated that pharmaceuticals with values for K_d lower than 300 L $kgMLSS^{-1}$ are considered to have low affinity to activated sludge (<10% elimination). Githinji et al. [15] analyzed the biosorption potential for AMX in synthetic wastewater and also obtained low $RR_{AMX,ads}$ (0.5 to 1.5%) in the first five hours for pH=7.5. In addition to this, the values of $RR_{AMX,ads}$ for the different AMX concentrations are fitted to the mathematical expression proposed by Fernández-Fontaina et al. [39] for the evaluation of $RR_{AMX,ads}$ as a function of the initial AMX concentration, K_d, X_{TSS} and concentration of AMX of the supernatant. If a K_d value of 1.06 L $kgMLSS^{-1}$ [37] was considered, $RR_{AMX,ads}$ ranged from 0.20 to 2.94%, which are similar to the values obtained in this research.

Despite the fact that the biological absorption is the main mechanism of removal of AMX in this research, Matsubara et al. [40] concluded that adsorption and biodegradation represented the largest removed fraction of AMX (68%) in a pre-

denitrification membrane bioreactor (A/O-MBR) system working at higher HRT values than that corresponding to this study (20-40 h and 4.8 h, respectively).

The trend observed in the present study differs from that of other β-lactam antibiotics, such as cefalexin and ampicillin [41]. In particular, cefalexin was significantly removed via biodegradation with a $RR_{AMX,biod}$ of around 95% in 5 h, and the major removal route for ampicillin was adsorption with 38.7% rapidly adsorbed by the activated sludge during the first 15 min. According to Li and Zhang [41], the removal routes for β-lactam antibiotics could vary greatly in wastewater treatment processes, possibly due to their different chemical structures, such as the variable side chains.

The negative correlation of $RR_{AMX,biod}$ with the concentration of AMX was in accordance with the results obtained by Baghapour et al. [7], as previously mentioned. In turn, the negative correlation of $RR_{AMX,ads}$ with pH was supported by Githinji et al. [15], who concluded that sorption decreased as the pH was raised.

Regarding the heterotrophic kinetic modeling, Calero-Díaz et al. [26] analyzed the effect of other antibiotic (ciprofloxacin) together with other pharmaceuticals (carbamazepine and ibuprofen) on the heterotrophic biomass of a membrane bioreactor system. In general, these authors worked at most favorable operation conditions with values of HRT of 6 h and X_{TSS} of 4551 mg L^{-1}. They obtained similar values of Y_H, although had values of K_M around 10-fold higher and values of $\mu_{m,H}$ about 3-fold higher than those corresponding to this work (Table 1). Thus, in presence of ciprofloxacin, there could be a certain inability to biodegrade carbon substrates (high K_M values), although the required time was lower than that in presence of AMX (high $\mu_{m,H}$ values). In addition, Monteoliva-García et al. [42] studied the effect of the same pharmaceuticals on the heterotrophic bacteria of a moving bed biofilm reactor-membrane bioreactor at

an HRT of 6 h and X_{TSS} of 5773 mg L^{-1}. These authors also obtained higher values of K_M than those corresponding to this research, remaining the rest of kinetic parameters similar.

Nevertheless, the values of Y_H corresponding to this research almost doubled previous results for nalidixic acid in a NIPHO activated sludge reactor [29]. Additionally, the values of $\mu_{m,H}$ obtained in presence of nalidixic acid (0.0080-0.0093 h^{-1}) were lower than those corresponding to AMX (Table 1). Regarding the values of K_M, these were higher (1.9073-6.3391 mgO_2 L^{-1}) for the nalidixic acid than those obtained in the present research for AMX. Thus, the generation of heterotrophic biomass per carbonaceous substrate biodegraded could be higher and heterotrophs could require less time to biodegrade the carbon source in presence of AMX. Moreover, in presence of nalidixic acid, heterotrophic biomass could have a possible inability to oxidize carbonaceous substrates, as indicated by higher K_M values. As indicated for ciprofloxacin, in addition to the influence of the kind of antibiotic, the different performance could be due to the operation conditions, which were less advantageous for the research carried out with nalidixic acid (HRT=2.8-3.8 h, T=12.6-14.8ºC).

The results obtained for b_H values were similar to those obtained by Calero-Díaz et al. [26] and Monteoliva-García et al. [42] analyzing ciprofloxacin, and Leyva-Díaz et al. [29] studying nalidixic acid.

The existence of statistically significant differences between $r'_{x,H,AMX}$ corresponding to 75 mg L^{-1} and the rest of concentrations could explain a possible change of trend for the highest concentration (75 mg L^{-1}) since $r'_{x,H}$ is lessened in presence of AMX. This concurred with the reduction observed for $r_{su,H}$ at 75 mg L^{-1} AMX concentration. The negative correlation of $r'_{x,H,AMX}$ with the concentration of AMX (Fig. 3c) could support

the most noticeable effect on $r'_{x,H,AMX}$ at the highest concentration (75 mg L^{-1}), as explained previously. Additionally, this confirms the trends observed for Y_H and $\mu_{m,H}$.

Consequently, AMX did not modify significantly the values of $r_{su,H}$ and $r'_{x,H}$. This could be explained by the existence of resistant bacteria and genes encoding resistance against certain β-lactams in WWTPs, according to Kümmerer [43]. In this regard, Bouki et al. [44] indicated that WWTPs provide an environment that has potential for the development and spread of antibiotic resistant bacteria, which could transfer resistance genes to resident bacteria, favoring their acclimatization. Likewise, Proia et al. [45] demonstrated that WWTPs could be hotspots for antibiotic resistance spread to nonresistant bacteria, with the presence of heterotrophic bacteria resistant to AMX, nalidixic acid, sulfamethoxazole and tetracycline. Zhang et al. [46] also identified and characterized antibiotic-resistant heterotrophic bacteria from WWTPs. In spite of this, as previously indicated, a more noticeable impact was detected for the highest concentration of AMX (75 mg L^{-1}). The reason could be based on the results obtained by Baghapour et al. [7], who stated that at high concentration gradient, AMX has more opportunities to be exposed to and penetrate into the cells that are responsible for biodegradation processes. In light of this, Matsubara et al. [40] analyzed the kinetic behavior of heterotrophic bacteria from a pre-denitrification membrane bioreactor (A/O-MBR) exposed to AMX concentrations ranging from 1 to 100 mg L^{-1}. These authors found that AMX level did not inhibit heterotrophic bacteria metabolism, although the highest tested concentration (100 mg L^{-1}) could affect the properties of sludge and respiration rate.

The values of $r_{su,H,AMX}$ are lower to those obtained in presence of ciprofloxacin by Calero-Díaz et al. [26] and Monteoliva-García et al. [42], which ranged from 183.97 to

192.88 mgO_2 L^{-1} h^{-1} and from 76.12 to 116.34 mgO_2 L^{-1} h^{-1}, respectively. This could be due to the higher X_{TSS} values, which were 2.3-fold [26] and 2.9-fold [42] higher than that corresponding to this research (Table S1). This fact could compensate the high values of K_M, add to the high values of $\mu_{m,H}$ in the research carried out by Calero-Díaz et al. [26]. Besides differences concerning operation conditions, the antibiotic structure could also support this behavior. In this sense, it should be highlighted that the amoxicillin is a β-lactam, which is mainly time dependent (slow antimicrobial activity), whereas the ciprofloxacin is a quinolone, which is concentration dependent in its activity. This could imply different minimum inhibitory concentrations [47]. In relation to other quinolone (nalidixic acid), the values of $r_{su,H,AMX}$ are similar to those obtained in presence of nalidixic acid by Leyva-Díaz et al. [29]. However, the values of $r'_{x,H,AMX}$ almost double those corresponding to the presence of nalidixic acid.

5. Conclusions

- Biological absorption played a major role in the removal of AMX, with removal rates varying from 11.06 to 87.13%. This behavior was different in relation to other β-lactam antibiotics in which biodegradation and biological adsorption were predominant. This was likely due to the different chemical structures. The operation variables that positively affected $RR_{AMX,abs}$ and $RR_{AMX,biod}$ were X_{VSS} and SVI, and the concentration of AMX exerted an important negative impact. Meanwhile, SRT, HRT, temperature and concentration of AMX were the variables with the highest influence on $RR_{AMX,ads}$, and it was inversely proportional to pH.
- The degradation rate for carbon source ($r_{su,H}$) and net heterotrophic biomass growth rate ($r'_{x,H}$) were not significantly affected by the presence of AMX. However, a

negative impact could be observed at the highest AMX concentration (75 mg L^{-1}), reducing the values of $r_{su,H,AMX}$ and $r'_{x,H,AMX}$ until 27.82 mgO_2 L^{-1} h^{-1} and 8.84 mgVSS L^{-1} h^{-1}, respectively, with the existence of statistically significant differences between this concentration and the rest of them. This could be due to higher probability of penetration of AMX into the cells at high concentration gradients.

- In absence of AMX, the variable with the highest influence on $r_{su,H}$ was the temperature, whereas SRT, pH and SVI were the variables with the greatest effect on $r'_{x,H}$. The presence of AMX negatively affected $r'_{x,H}$, which could justify the most noticeable impact at 75 mg L^{-1} AMX concentration. In light of this, the trend was opposed for $r_{su,H}$, which could suggest an acclimatization behavior against the shock caused by AMX for concentrations from 15 to 60 mg L^{-1}.

CRediT authorship contribution statement

Laura García: investigation, writing original draft. **Juan Carlos Leyva-Díaz:** visualization, writing original draft. **Eva Díaz:** writing review & editing, project administration. **Salvador Ordóñez:** writing review & editing, funding acquisition.

Declaration of Competing Interest

The authors declare no financial or commercial conflict of interest.

Acknowledgements

Authors would like to acknowledge ACUAES (public consortium for the management of MWWTPs in Spain) and Eva María Álvarez for providing us the required samples from the WWTP of Villapérez, Asturias (Spain). The authors gratefully acknowledge financial support from the Asturian Government (IDI/2018/000116).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:

Nomenclature

b_H	decay coefficient for heterotrophic biomass
$b_{H,AMX}$	decay coefficient for heterotrophic biomass in presence of AMX
$b_{H,n/AMX}$	decay coefficient for heterotrophic biomass in absence of AMX
K_M	half-saturation coefficient for carbon source
$K_{M,AMX}$	half-saturation coefficient for carbon source in presence of AMX
$K_{M,n/AMX}$	half-saturation coefficient for carbon source in absence of AMX
$m_{AMX,abs}$	mass of AMX removed via absorption
$m_{AMX,ads}$	mass of AMX removed via adsorption
$m_{AMX,biod}$	mass of AMX removed via biodegradation, not discarding other chemical routes
$m_{AMX,i}$	initial mass of AMX added to the sludge
$m_{AMX,dis}$	mass of AMX dissolved in the supernatant after the respirometric test
$RR_{AMX,abs}$	removal rate for AMX absorption
$RR_{AMX,ads}$	removal rate for AMX adsorption
$RR_{AMX,biod}$	removal rate for AMX biodegradation
$r_{su,H}$	degradation rate for carbon source
$r_{su,H,AMX}$	degradation rate for carbon source in presence of AMX
$r_{su,H,n/AMX}$	degradation rate for carbon source in absence of AMX
$r'_{x,H}$	net heterotrophic biomass growth rate
$r'_{x,H,AMX}$	net heterotrophic biomass growth rate in presence of AMX

$r'_{x,H,n/AMX}$ net heterotrophic biomass growth rate in absence of AMX

R_S dynamic oxygen uptake rate

X_{TSS} Mixed liquor total suspended solids

X_{VSS} Mixed liquor volatile suspended solids

X_H concentration of heterotrophic biomass

X_T total biomass concentration

Y_H yield coefficient for heterotrophic biomass

$Y_{H,AMX}$ yield coefficient for heterotrophic biomass in presence of AMX

$Y_{H,n/AMX}$ yield coefficient for heterotrophic biomass in absence of AMX

Greek symbols

$\mu_{m,H}$ maximum specific growth rate for heterotrophic biomass

$\mu_{m,H,AMX}$ maximum specific growth rate for heterotrophic biomass in presence of AMX

$\mu_{m,H,n/AMX}$ maximum specific growth rate for heterotrophic biomass in absence of AMX

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