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Effect of amoxicillin releases on the aerobic activity of heterotrophic biomass

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ABSTRACT

Due to its high use, amoxicillin (AMX) contributes in a large measure to the pollution of aquatic environment. Unfortunately this emerging pollutant has not been covered by the release standards. There is little information regarding the AMX effect on heterotrophic biomass of the wastewater treatment plant (WWTP), which are not designed to degrade them, causing possible dysfunction. The novelty in this study is the application of LFS respirometer as a fast, efficient and robust means to evaluate the AMX effect on heterotrophs response, allowing the determination of inhibition rates by simulating the biological processes taking place in conventional WWTP. Three types of activated sludge and an easily biodegradable synthetic substrate based on sodium acetate at 300 mg_{COD}/L considering the ratio S_0/X_0 = 0.04 g_{COD}/g_{VSS}, were used. Respirometry showed quite different and low inhibition rates of heterotrophic where the maximum reduction in $Y_{O/S}$ rate was 30.6%, 22% and 20% for 75, 120 and 150 mg_{AMX}/L, respectively explaining that the heterotrophic biomass adapted to the substrate and to AMX showed a fairly significant resistance to inhibition compared to non-acclimated one. Heterotrophs biomasses are less sensitive and quick to adapt because they were inhibited by 35% at 150 mg_{AMX}/L, on other, biomass contained autotrophes, which were inhibited up to 35% in 25 mg_{AMX}/L.

Keywords: Amoxicillin antibiotic; Inhibition; Respirometry; Heterotrophic biomass activity; Activated sludge

1. Introduction

Currently, the challenge of providing safe drinking water is increasing with the growing population [1]. The pollution induced by antibiotics and their by-products originating from domestic, hospital and industrial activities, has been recognized as one of the most serious threats to public health in the 21st century, mainly due to a continuous release into aquatic environments and ecosystems where they accumulate. Currently they are not covered by regulation or environment limits unlike other micropollutants. Therefore they are considered as emerging pollutants [1–4]. This increases the risks of development, maintenance, transfer, spreading and transmission of antibiotic resistance genes (ARGs) to treatment and drug over the long term, hence an irreversible effect favored by low concentrations and having serious impacts on the ecosystem where the physiological function of wastewater treatment plant (WWTP) microbiota [5–8] may be disrupted. There is evidence that these ARGs can be transmitted to humans [9–11]. However, up to now, it is difficult to completely remove antibiotics by conventional biological system where during such treatment, resistant genes can spread via the activated sludge process [12,13]. Despite this, biological treatment systems remain the most promising for the treatment of wastewater containing pharmaceuticals compounds, due to their cost effectiveness while being friendly to the environment [14]. Among the antibiotics, β-lactams are the most popular and represent more than 65% of the global antibiotic market, with annual use reaching 100–200.000 tons

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worldwide [15]. Mainly developed to achieve a biological effect, these antibiotics, are often recalcitrant, toxic [16] and have a high wastewater concentration in pharmaceutically active compounds (PhACs), often exceeding 100 mg/L [17]. Amoxicillin (AMX) is among the β-lactams, widely spread for several decades as more important micro-pollutants [18]. It is a broad-spectrum amino-penicillin antibiotic, effective against many gram-positive and gram-negative microorganisms [19–23] and is widely used as an inhibitor in modern human and veterinary medicine, due to its chemical structure, polarity, activity level, antibiotic specifications and environmental sustainability.

The massive production of AMX is the reason of its presence in different water types and in wastewater treatment plant (WWTP) [24–28]. AMX, like other biologically active substances, influences the bacterial biomass, can disrupt penicillin binding proteins and interferes with cell wall biosynthesis during reproduction, ultimately leading to microbial osmotic rupture [29], causing disruptions and this may occur within purifying dysfunctions cycles. The AMX was selected for this purpose for two reasons: (a) less than 30% of the used dose are not metabolized by the body, meaning that this molecule excreted by the body is present in an active form rather than a by-product. This allows to simulate experimental tests with the use of direct active ingredients; b) AMX is used to treat a variety of infection, which makes it a candidate to be included in the medical supplies [30,31]. Various methods are known for the evaluation of inhibition in activated sludge, like enzymatic inhibition, respiratory rate inhibition, inhibition of the activated sludge process efficiency, activated sludge micro-fauna inhibition, influence on bacterial kinetics of activated sludge, inhibition of cell viability by plate counting. The response of the viable heterotrophic biomass was carried out by combining batch experiments with respirometry under normal and inhibition conditions to determine the AMX attenuation to heterotrophic activity. Currently, respirometry is considered as the most rapid, sensitive, and economical dominant technique for evaluating AMX toxicity to heterotrophic biomass activity before and after their use [32,33]. Oxygen uptake rate (OUR) profiles per unit time and volume were used to assess and identify the chemical oxygen demand (COD) fraction and inhibition percentage [34–36].

Many physical and chemical methods have been suggested for the AMX removal from aquatic environments. Biodegradation and adsorption have been the main removal routes, but several studies [37] have shown that the removal of these molecules in conventional WWTP is often incomplete and hence may inhibit the biological activity [38–43], so that they are discharged into surface waters and then enter different aquatic compartments, thus biodegradation leads to variable transformations depending to the molecules of a relatively weak purification would be only 5% [44] to more 99% of elimination. Usually they leave the WWTPs almost unchanged. Effective methods are very expensive and present many performance problems such as a membrane bioreactor (MBR) [24]. However, AMX has been almost completely removed by simple and inexpensive activated sludge processes such as SBR process (sequencing batch reactor) at the end of each cycle [45]. The AMX removal was not the objective of this study, but focus was on the inhibitory effect identification and the heterotrophic biomass response in the case of their presence in WWTP. Although antibiotic toxicity could inhibit the functional microbial community to varying degrees, the damage could be controlled by complex factors [46] that influence the degree of inhibition: VSS and TSS concentration, pH, solubility and inhibitor concentration, properties and bioavailability of inhibitor, the inhibitor form (dissolved or particulate, free or complex, sorbed or not on sludge) [47–49], the species present in the effluent and the liquor, SRT and hydraulic retention time (HRT), acclimation or not of the biomass, the biomass/toxic exposure time, the initial S_0/X_0 ratio which influences the AS biocenosis [50–52]. The releases of this antibiotic are of a concern because they would contribute to the maintenance and phenomenon of the emergence and dissemination of resistant germs, as well as to antibiotic therapy failures, due to this inappropriate and inconsiderate use and to the residence time in the environment coupled with the adaptive capacity of microorganisms and the possibilities of genetic transfer. Although the activated sludge processes favor the total or partial antibiotics biodegradation generally provide an ideal medium for the ARGs presence, as well as for their transfer [53]. An easily biodegradable substrate, acting as an energy source and electron donor, could enhance the biotransformation and refractory organic pollutants mineralization, including antibiotics [54–56]. The appropriate easily biodegradable substrate can promote the antibiotics detoxification such as AMX [57] by optimizing bacterial community structures and altering functional gene abundance [58–60]. In addition, an easily biodegradable substrate strategy has been used to activate functional enzymes and to accelerate the refractory organic matter biodegradation [61–64]. But it is important to know how an easily biodegradable substrate affect the ARGs production and understand their role in the micro-pollutants biodegradation. An easily biodegradable substrate like sodium acetate, may add a positive role in shortening the microbial acclimation period and stabilize the effluent quality. If sodium acetate reaches 100 mg/L in influent, the AMX removal will be almost 100% (after a long contact time).

The present study, investigated the AMX effect, on the active heterotrophic biomass response fraction in non-acclimated AS (substrate oxidation inhibition) and on others acclimated AS (anabolic pathways inhibition). Since the shock produced by AMX was different, this biomass was generally approximated by the viable fraction responsible for the biological process by the amount of TSS or VSS which could be easily determined [65,66]. However, little research had been carried out on AMX effect on heterotrophic biomass response and on the biodegradation. Adsorption of this molecule on bacterial activated sludge flocs, was generally not significant and therefore it was not considered, being out of the scope of the present study.

2. Materials and methods

2.1. Respirometry setup

The respirometry built for the present study is simple and based on a batch reactor; the installation is an assembly

of measuring and sampling instruments (Fig. 1). The respirometric experiments were carried out on an applied flow-gas/static-liquid type (LFS respirometry). The device is an open vessel, which facilitates the addition of synthetic substrate or the targeted inhibitor, so that the oxygen supplied to the respirometry is not a limiting factor. However for this case, it was applied for the number of injections introduced in the same experiment requiring a fairly long time to complete a single test.

A mass balance for oxygen in the respirometry can then be rewritten as [82]:

$$\frac{d\left(V_L S_0\right)}{dt} = Q_e S_{\mathrm{Oe}} - Q_S S_O + V_L K_L a\left(S_{\mathrm{Os}} - S_O\right) - V_L \mathrm{OUR}_T \quad (1)$$

where V_L: volume of liquid phase in the respirometer (L); S_O: concentration of dissolved oxygen (DO) in the liquid phase (mg/L); S_{Oe}: DO concentration at the inlet of the respirometer; S_{Os}: Saturation concentration of dissolved oxygen in the liquid phase (mg/L); $K_L a$: oxygen transfer coefficient in the liquid phase (h^{-1}); Q_e and Q_s: liquid flow at the inlet and outlet of the respirometer respectively (L/h); OUR_T: total oxygen uptake rate (mg/L·h)

In an LFS respirometry, [Eq. (1)] is reduced to both Eqs. (2) and (3) [73,82]:

$$\mathrm{OUR}_{\mathrm{exo}} = K_L a\left(S_{\mathrm{end}} - S_O\right) - \frac{d\left(S_O\right)}{dt} \quad (2)$$

$$\mathrm{OUR}_{\mathrm{T}} = K_L a\left(S_{\mathrm{OS}} - S_O\right) - \frac{d\left(S_O\right)}{dt} \quad (3)$$

$$\mathrm{OUR}_{\mathrm{T}} = \mathrm{OUR}_{\mathrm{exo}} + \mathrm{OUR}_{\mathrm{end}} \quad (4)$$

2.2. Pharmaceutical synthetic effluent

Amoxicillin is worldwide the most commonly used antibiotic of the β-lactam family. It was discovered by Alexander Flemming in 1928–1929 [67], and was obtained by fermentation in the absence of penicillin G-specific side chain precursors [68]. AMX is used to treat various infections caused by bacteria in the ear, lung, nose, teeth, urinary tract and skin infections. It is also used before surgery to prevent infections [69]. AMX has a good diffusion throughout the organism because it binds 17%–20% to plasma proteins. Due to its very low lipophilicity, it is not able to diffuse inside the host cells and therefore cannot be active on intracellular bacterial forms.

From the moment of its administration, AMX undergoes simultaneous distribution and elimination phenomena. The gradient concentration between the intra- and extravascular medium favors the movement of this molecule from the administration space to the rest of the pharmacokinetic system in comparison to the elimination process. This results in a rapid drop in plasma drug concentrations, known as the distribution phase. Gradually, this gradient concentration cancels itself out and reaches a state of pseudo-equilibrium. This results in a slowing of the drop in plasma concentrations (elimination phase), which is the process of irreversible transfer of the PhACs from the measurement site, the plasma, to the outside of the pharmacokinetic system under study. It can be achieved in two ways, either by excretion or by metabolism, which is elimination by an enzymatic process that will chemically transform the PhACs into a substance that can be more easily excreted. The organs responsible for the elimination of a PhACs are called emunctory organs (kidneys and liver). The skin, lungs and intestine are other possible routes of elimination. AMX is mainly excreted from the kidney using tubular secretion mechanisms. Its excretion in the bile has been

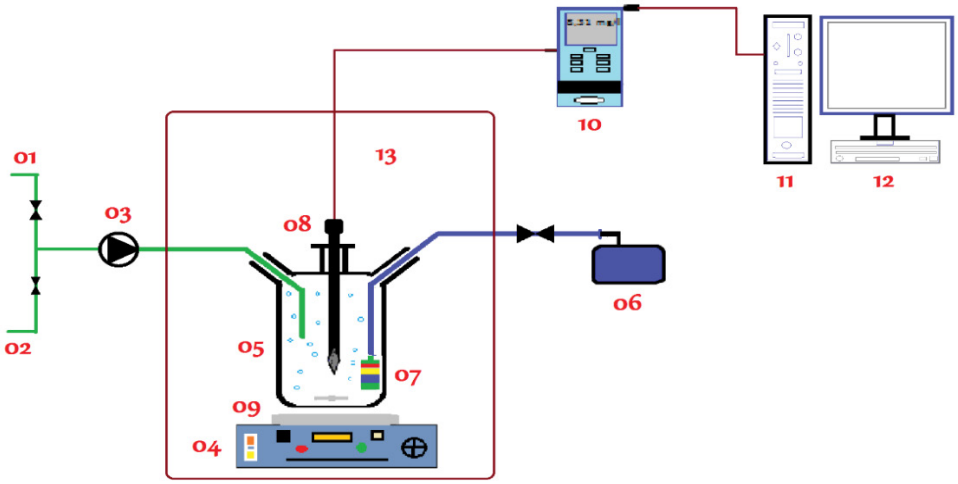

Fig. 1. Scheme of LFS respirometric cell. 1-Supply system for different injections (substrate, inhibitor). 2-Racking system for sampling (emptying, samples). 3-Control system (Valves). 4-Agitation system (agitator plate). 5-Bioreactor (transparent glass batch reactor with a volume of 0.6 L). 6-Aeration system (air injection with a constant flow rate of 150 L/h·min). 7-Air diffusion system (fine air bubble diffuser made of plastic and/or ceramic). 8-Multi-use probe (for different measurements: DO, pH, TDS, T°, salinity, conductivity). 9-Homogenization system (magnetic stirring bar). 10-Multi-parameter measuring system. 11-Data acquisition and recording system. 12-Data processing system. 13-Incubation medium of installation (thermostatic chamber set at 20°C ± 2°C).

Table 1
Physical–chemical properties of the AMX antibiotic [24,71,72]

Therapeutic class	Antibiotic
Family	β-lactams
IUPAC name[a]	α-amino-p-hydroxybenzyl penicillin
Synonyms	Amox; AMC; AMX; Amoxicillin trihydrate; Amoxicillin anhydrous; D-amoxicillin; p-hydroxyAMP
Gross formula	$C_{16}H_{19}N_3O_5S$
Raw formula of the oral form	$C_{16}H_{19}N_3O_5S{\cdot}3H_2O$
No. CASRN[b]	26787-78-0
Molar mass (g/mol)	Amoxicillin: 365.40; Amoxicillin trihydrate: 419.41
Physical characteristics	Solid or liquid, white to off-white crystalline powder, penicillin-type odor
Solubility in water (g/L)	3.9 (around 3.43 g/L)
Solubility in organic solvents	
Methanol	7,500 mg/L
Ethanol	3,400 mg/L
Melting point	194°C
Boiling point	743.2°C at 760 mmHg
Flash point	403.3°C
$\log K_{OW}$[d]	0.87
pKa[c]	3.39, 6.71, 9.41
λ_{max} (nm)	233

[a]International Union of Pure and Applied Chemistry;
[b]Chemical Abstract Services Registry Number;
[c]Acid dissociation constants;
[d]Octanol/water partition coefficient.

demonstrated in humans and animals where it is eliminated in the same administered form [70].

AMX is a semisynthetic drug with a broad spectrum of activity. It inhibits the interactions between the linear peptidoglycans chains which act by disturbing the cell walls synthesis of bacteria during reproduction, it is a β-lactam antibiotic, having a very short residence time in the stomach and it degrades in gastric acid.

2.3. Synthetic wastewater substrate

The synthetic effluent used in this study contained the substrate sodium acetate as a carbon source and ammonium chloride as a nitrogen source as well as other necessary micronutrients for bacterial growth (Table 3). The products used are easily biodegradable by nature in order to ease their degradation and rapid acclimation since they have a very low latency phase. The established injections will be performed, while the COD concentration was 300 mg/L.

Sufficient easily biodegradable substrate could be attributed to the functional genes development that maintained microbial activity and developed an optimized microbial community to enhance the micro-pollutants

Table 2
Results of some previous studies on AMX removal [21,22,24]

Operational condition	HRT (h)	AMX removal efficiency %
Up-flow anaerobic sludge blanket (UASB)	23.2	21.6
Up-flow anaerobic sludge blanket (UASB)	23.5	20.2
Novel micro-aerobic hydrolysis acidification reactor (NHAR)	9.3	20.4
Cyclic activated sludge system (CASS)	14.9	68.2
Biological contact oxidation tank (BCOT)	14.9	80.6

Table 3
Composition of the synthetic substrate used in the current study [73,74]

Constituents	Concentration (mg/L)
Carbon source: $C_2H_3O_2Na$ (sodium acetate)	2.564
Nitrogen source: NH_4Cl (ammonium chloride)	1.492
Micronutrients	
NH_4Cl–N	0.0068
$FeCl_3$	0.0023
$MgSO_4$	0.166
$CaCl_2$	0.412

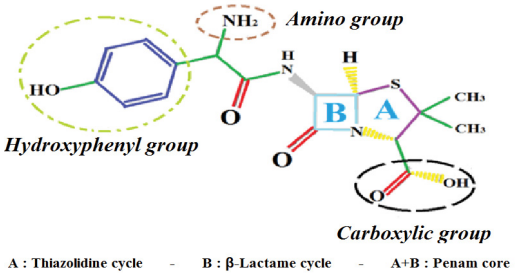

Fig. 2. AMX semi-developed structure identifying its ionizable groups, nucleus β-lactams cycles [24,74].

biodegradation [56,75]. The easily biodegradable substrates use had developed optimized microbial community structures that enhanced and stabilized AMX biodegradation. Consequently, the microbial cell walls appeared cloudy and severely decomposed. Such cell wall decomposition was significantly mitigated when the easily biodegradable substrate had optimized functional microorganisms. Indeed, the integrity of cell morphology with the easily biodegradable substrate was contributed by the increased coding genes expression, penicillin-binding proteins and up-regulation of β-lactamases as well as down-regulation of enzymes that inhibited peptidoglycan biosynthesis. Sodium acetate had an up-regulates β-lactam damaging enzymes [60] within the first hour. The sodium acetate addition contributed to the abundant ARG removal and resistant pathogenic bacteria. Such ARG might cause a serious public health challenge,

worldwide [76,77]. The microbial community structure was affected by environmental factors and this could determine the ARGs production and could promote or mitigate bacteria harboring ARGs as well as modify ARG profiles [78,79]. AMX removal efficiency increased significantly with increasing supplementation of easily biodegradable substrate. These improvements were attributed to the microbial activity promotion and functional microorganism enrichment [56]. Overall, information on the removal and the fate of antibiotics in WWTPs was still limited.

2.4. Sludge inoculum

In order to evaluate the effect of the introduction of AMX on a variety of activated sludge (AS), three types of sludge were used. The first inoculum was an AS in its raw state taken from the aeration tank of the local urban WWTPs. The two other inocula used in the different experiments carried out were taken from the two adaptation bioreactors previously started up for 38 d where the first one aimed to adapt the microorganisms to the synthetic substrate and the second-one to the synthetic substrate with AMX (Fig. 3). The different characteristics of inocula taken from the treatment plant or the adaptation bioreactors are grouped in (Table 4).

An increase in the salinity of inocula was observed in the two adaptation bioreactors caused mainly by the nature of the used synthetic carbonaceous substrate, which was in the salt form. The adaptation phase was carried out gradually with a time step varying between 5 and 8 d and increasing substrate concentrations and AMX reaching 11 and 1.5 g/L, respectively (Fig. 4). Several parameters were carried out to monitor the operating status of the two adaptation bioreactors using the Multi-parameter measuring system allowing the following of temperature (°C), pH, DO (mg/L), salinity (%), conductivity (μS/cm) and total salts (mg/L) as well as microscopic visualizations in fresh or stained state using a microscope with variable magnification (Infinity, Optech) (Fig. 3). The sludge adapted to the substrate and the inhibitor, presented a more visual state of compaction than the other two sludges, where the flocs were more open, with an irregular shape.

2.5. Sludge preparation for the various respirometric tests

Two main techniques designated by the endogenisation process had been developed for the preparation of AS in respirometric tests. These sludges taken from the different compartments of the WWTP biological line, mainly from biological basins, were either subjected to continuous aeration [80,81] or are washed. The importance of these preparations was to get rid of the exogenous substrate recovered with the AS during sampling without considerably disturbing the initial state of sludge activity. It is important to note that the sludge washing technique was mainly used for sludge with good settling capacities, presenting an SVI (sludge volume Index) ranging from 100 and 150 mL/g, otherwise sludge loss might occur during washing and if the sludge had SVI higher than 150 mL/g, the technique of continuous aeration was more recommended. Zamouche-Zerdazi et al. [82] used the washing technique for a healthy sludge with a filamentous index (FI) varying between 1 and 3 and the technique of continuous aeration for 24 h, for a bulking sludge with very poor settling capacities and an FI varying between 4–5. Endogenous aeration requires a fairly long period of time, especially for those using the continuous aeration technique, where the time can

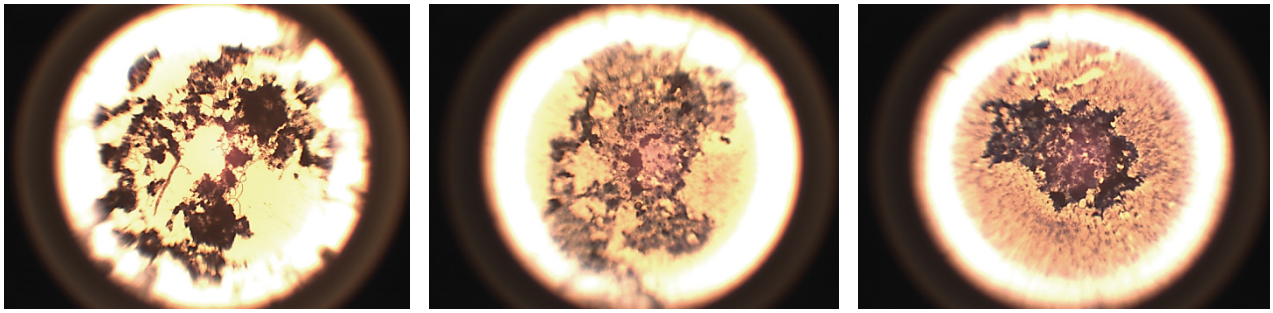

Fig. 3. Microscopic visualization of the different activated sludge used. (a) Raw activated sludge, (b) sludge adapted to substrate, and (c) sludge adapted to AMX + substrate.

Table 4
Main characteristics of the inocula used in the current study

	pH	Salinity (%)	Conductivity (μS/cm)	DO (mg/L)	Temp. (°C)	Mineralization ratio (%)	Substrate adaptation	AMX adaptation
AS1	7.41 ± 0.10	0.65 ± 0.05	1,408.5 ± 55.5	4.22 ± 0.09	–	41–45	(–)	(–)
AS2	6.89–8.84	0.7–27.6	1,569–43,200	3.04–8.20	13.1–22.6	30–39	(+)	(–)
AS3	7.13–8.80	0.7–22.6	1,567–35,900	0.11–8.07	13.6–23.7	36–40	(+)	(+)

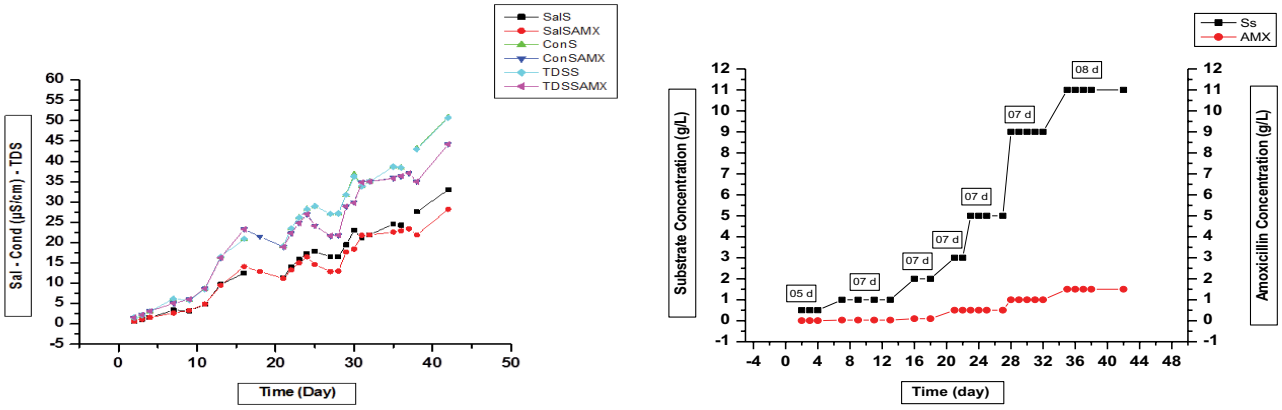

Fig. 4. Salinity evolution and the program improved in the adapted reactors.

vary from a few hours to 5 d. In order to carry out this preliminary preparation of the AS, an optimization of the operating conditions of the washing technique had been carried out in previous studies, to mention the time and number of washes. The various inocula would undergo a single washing and then continuous aeration at 20°C for a period not exceeding 3 h, in order to reach an endogenous state characterized by a plateau in the temporal DO measurements [73].

3. Results and discussion

3.1. Determination of the oxygen transfer coefficient (K_La)

The K_La must be evaluated according to the operating conditions imposed in the respirometric tests (flow rate, aeration mode, stirring speed, reactor geometry). The estimation methodology is based on the exploitation of the aeration and/or deaeration curve obtained after a disturbance imposed on the DO concentration in endogenous condition (the disturbance is imposed after reaching the apparent endogenous state as a baseline when monitoring the oxygen concentration) [83]. Linear regressions for the different slopes estimation will be performed in the first part of the evolution of the oxygen concentration of the aeration or deaeration curve and the liquid phase K_La can be estimated using the equations obtained by establishing mathematical developments in each considered situation.

By exploiting only, the deaeration curve (part B/slope 1), the K_La will be estimated by Eq. (5), on the other hand by exploiting the deaeration and aeration curves (part B and D/slope 1 and 2), K_La will be determined by Eq. (6) and finally by exploiting the aeration curve (part D/slope 2) K_La will be estimated by Eq. (7), (Fig. 5) [73,82].

$$K_La = \frac{\mathrm{OUR}_{\mathrm{end}}}{S_{\mathrm{OS}} - S_{\mathrm{end}}} \tag{5}$$

$$K_La = \frac{\text{Slope 02} - \text{Slope 01}}{S_{\mathrm{OS}} - S_{\mathrm{end}}} \tag{6}$$

$$\ln\left[\frac{S_{\mathrm{end}} - S_O}{S_{\mathrm{end}} - S_O\left(t=0\right)}\right] = K_Lat \tag{7}$$

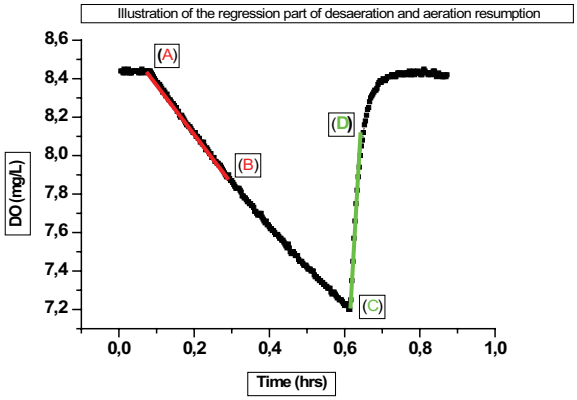

Fig. 5. Regression part illustration of deaeration and aeration resumption for the K_La estimation.

The K_La determination must be meticulously carried out since this parameter conditions the respirometric responses in continuous aeration for the different injections. These three K_La determination approaches will be applied to an experiment carried out for a S_0/X_0 ratio equal to 0.04 g_{COD}/g_{VSS} (Fig. 6). According to the obtained results and grouped in (Table 5), the first development seems to underestimate K_La (low values compared to the two others) (Fig. 7), because it only considered the deaeration phase in oxygen transfer determination. In this phase, the DO consumption were very fast and only due to the microorganisms endogenous respiration. However, the second development considers the two phases of the respirometric response (deaeration and aeration) where all the consumption oxygen transfer was considered. The oxygen transfer from the gaseous to the liquid phase was conditioned by the aeration rate and the stirring speed applied in the respirometry (operating conditions) and from the liquid to the solid phase conditioned by the microbial activity. Finally the third development only considered the aeration phase, masking the endogenous microbial activity. This development can only be exact in the determination case of K_La in clear water. The results considered in the present study will be those allowing the K_La determination using the second development.

It is important to note that the maximum exogenous respiration after the substrate injection is sometimes lower

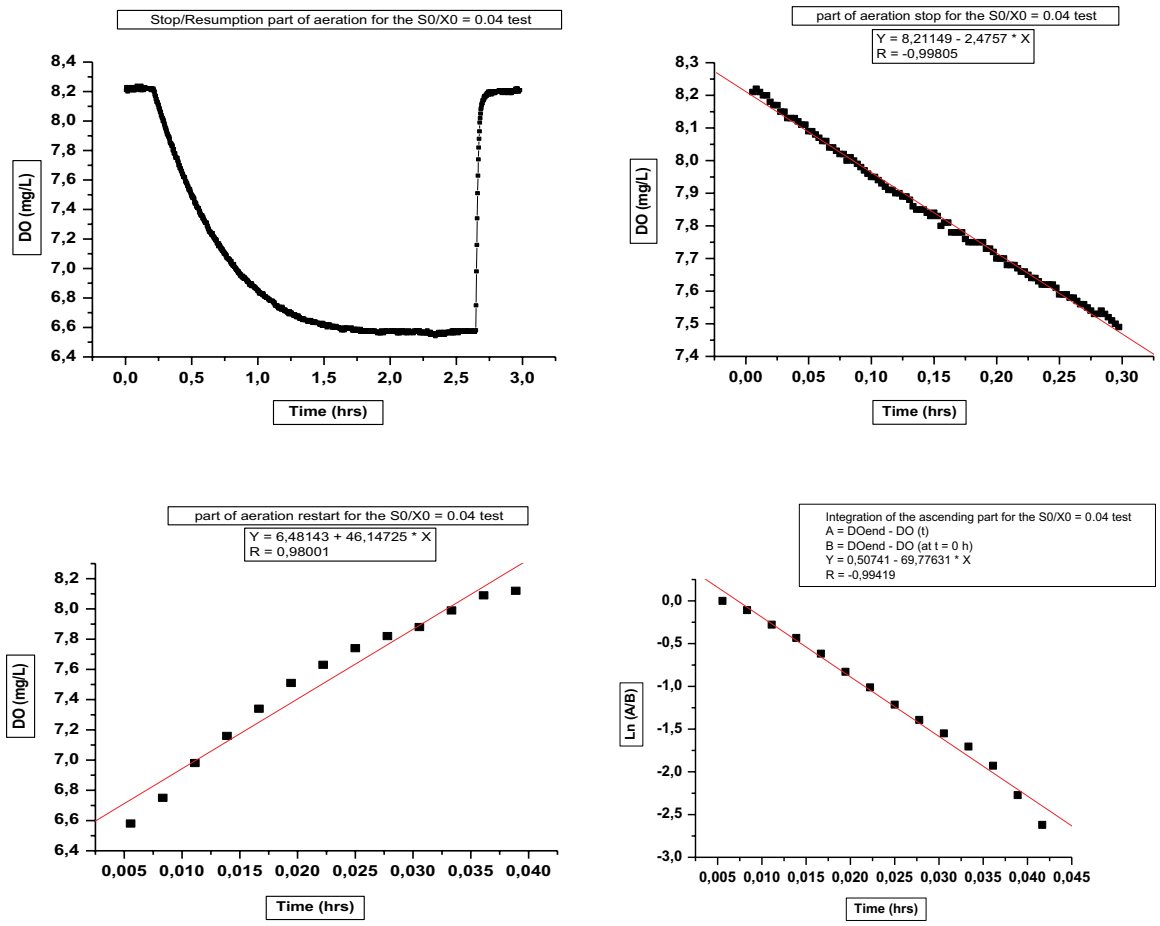

Fig. 6. Application of the three mathematical developments for the K_La determination and for a S_0/X_0 ratio = 0.04 g_{COD}/g_{VSS}.

Table 5
Respirometric responses characteristics using K_La determined with the three mathematical approaches (S_0/X_0 = 0.04 g_{COD}/g_{VSS})

S_0/X_0: 0.04 g_{COD}/g_{VSS}	OUR_{end} (mg/L·h)	K_La (h^{-1})	OUR_{exomax} (mg/L·h)	Q_{exo} (mg/L of reactor volume)	Q_T (mg/L of reactor volume)	T_s (h)
Linear descending response	2,475	2,153	1,163	2,547	12,409	3,983
Linear descending and ascending response		50,197	27,106	59,376	69,237	
Linear ascending response		69,776	37,679	82,535	92,397	

than the endogenous respiration (Table 5), in the case where only the deaeration phase is considered. This confirms that the first approaches for K_La determination underestimates it.

3.2. S_0/X_0 ratio fixing in respirometric tests

In order to determine the heterotrophs oxygen consumption rate "OUR_{exoH}", it is necessary to block the autotrophs respiration by using nitrification inhibitors such as allylthiourea (ATU) and 2-chloro-6-(trichloromethyl) pyridine (TCMP). The applied dose depends on the autotrophs concentration in the AS. For a mixed population, the fraction of nitrifying organisms in the AS culture increases with the N/COD ratio of the wastewater, since heterotrophic bacteria can be competitive for the nitrogen substrate they consume for their anabolic requirement. In the present study a dose of 20 mg/L of ATU was used in the respirometric tests to an estimated carbonaceous substrate oxidation [82] and the inhibitor was introduced after the endogenous respiration and the K_La determining step, for all established tests, using the inocula AS1, AS2 and AS3.

A contact time of 20 min between the nitrification inhibitor and the biomass was fixed before any further introduction of AMX and the synthetic substrate into the batch respirometry. The used substrate quality, the inocula and the operating conditions were very important to set in the respirometric tests, particularly the initial ratio, between the substrate and the biomass of the mixture, S_0/X_0.

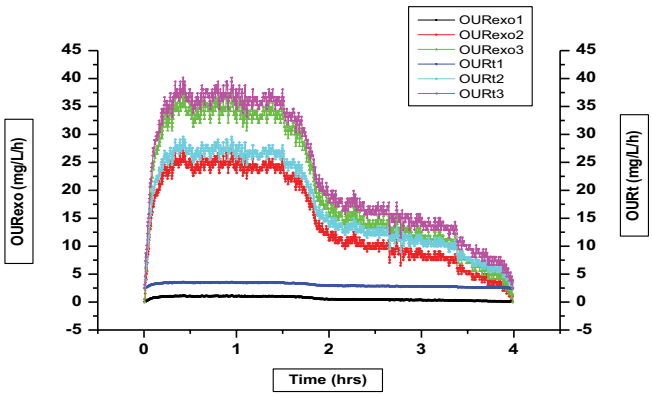

Fig. 7. Exogenous and total respiration obtained using the three K_La determined using the three mathematical developments for the S_0/X_0 ratio = 0.04 g_{COD}/g_{VSS}.

This ratio determined the respirometric response, influenced the metabolic processes involved in the degradation, the kinetic parameters identification and the easily and slowly biodegradable compartments dissociation.

In this study, the S_0/X_0 ratios was obtained by varying the injected synthetic substrate and the AS pre-treated volumes in the respirometric cell and is expressed in g_{COD}/g_{VSS}. In the batch respirometric studies, a low S_0/X_0 ratio less than 0.2 g_{COD}/g_{VSS} was used, in order to minimize the experiment duration and to carry out several successive substrate injections, thus avoiding the significant growths generation during successive experiments carried out in the same respirometric test, which could confuse the inhibition study, since the production of a healthy biomass (uninhibited) would take place.

Many authors consider that these low ratios allow the obtention of the kinetic constants representative of the initial biomass [82]. Experiments were carried out on heterotrophic biomass and for different S_0/X_0 ratio using the AS1 inoculum. The different experiments results are summarized in the (Table 6); the (Fig. 8) illustrates the variation in maximum heterotrophs exogenous respiration as a function of the degradation time for each S_0/X_0 ratio tested.

Above S_0/X_0 equal to 0.06 g_{COD}/g_{VSS}, the experiment seemed to be very cumbersome since the recommended time for the degradation of the synthetic substrate (T_s) was very extended, limiting the performance of successive injections and allowing to quantify the effect of PhACs waste at different concentrations on the heterotrophs activity, bearing in mind that each inhibition test would be preceded by its control test (without inhibition). So these experiments were carried out for not adapted sludge to the substrate or to AMX (AS1) and times would certainly be reduced for the inocula AS2 and AS3 after adaptation. By varying the S_0/X_0 ratio from 0.01 to 0.23 g_{COD}/g_{VSS}, the maximum heterotrophs respiration reached 53.1133 mg/L·h (Fig. 7).

An S_0/X_0 ratio of 0.04 g_{COD}/g_{VSS} would be set in all heterotrophs inhibition tests of AS1, AS2 and AS3 inocula, although the heterotrophs maximum respiration ($OUR_{exo,maxH}$) without inhibition did not reach the encountered values for AS, ranging between 30–40 mg/L·h (Table 6), but many researches have been established in close reports for the

Table 6
Parameters characterizing respirometric responses for different S_0/X_0 ratios

	S_0/X_0 (g_{COD}/g_{VSS})	K_La (h^{-1})	Q_{exoH} (mg/L)	T_s (h)	$OUR_{exomaxH}$ (mg/L·h)
COD = 300 mg/L	0.01	19.274	1.614	0.681	3.469
	0.02	63.426	12.493	1.356	15.222
	0.03	67.154	19.441	1.856	14.773
	0.04	36.164	14.831	2.253	10.125
	0.06	28.220	16.076	2.869	7.6195
	0.09	56.992	82.720	7.728	19.947
	0.15	28.423	73.984	10.050	15.348
	0.23	64.016	375.967	23.672	53.133

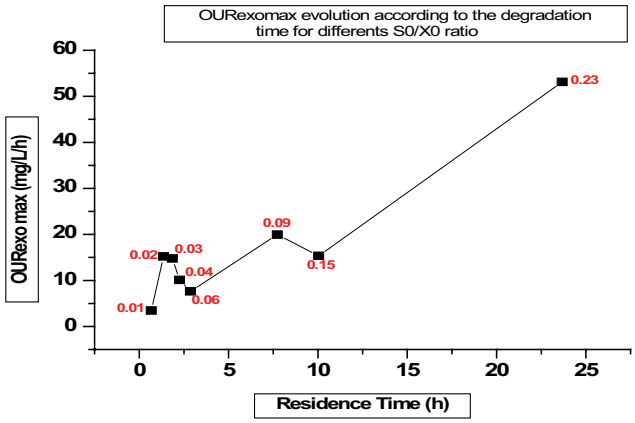

Fig. 8. Maximum exogenous respiration of heterotrophs according to the degradation time for different S_0/X_0 ratios (AS1).

estimation of biokinetic constants, for instance, Daniele et al. [84] evaluated the heterotrophic biokinetic constants using LFS respirometric batch tests established at a ratio S_0/X_0 of 0.05 g_{COD}/g_{VSS} to study the contribution of the supports in the treatment by MBBR (moving bed biofilm reactor).

3.3. AMX effect on heterotrophs microbial activity in aerobic conditions

The AMX effect on the heterotrophs activity in aerobic conditions was mainly evaluated by estimating the conversion efficiency of heterotrophs noted (Y_H). It should be remembered that in discontinuous cultures, similarly to continuous ones, a portion of the substrate (S) was converted into new cells (X) characterized by the fraction Y_H and a portion was oxidized to provide the energy characterized by the fraction ($1–Y_H$) noted $Y_{O/S}$ [84]. Y_H was determined using the following equation [84]:

$$Y_H = 1 - Y_{o/s} = 1 - \frac{Q_{TH}}{\Delta S_S} \quad (8)$$

where Q_{TH}: The amount of DO consumed by the heterotrophs (calculated by integration of respiration rate profile); ΔSs: The amount of carbonaceous substrate removed, expressed in CODs.

This parameter could only be assessed by inhibiting the autotrophs activity as mentioned above; the effect was quantified on the three different inocula. The effect on activity could be evaluated by estimating either the slopes percentage reduction (*P*) (the parameter that can be measured most rapidly) of the respirometric peaks, in the presence and absence of the PhACs [Eq. (9)], the reduction percentage in the total oxygen consumed quantity (Q_T) [Eq. (10)] [84], or autotrophs (Q_{TA}) and heterotrophs (Q_{TH}) oxygen consumed quantity in the presence and absence of inhibitors.

$$P = \frac{(\text{pente})_0 - (\text{pente})_1}{(\text{pente})_0} \times 100 \quad (9)$$

$$R_T = \frac{Q_{T0} - Q_{T1}}{Q_T} \times 100 \quad (10)$$

Endogenous respiration for AS1 varied between 3.166 and 11.741 mg/L·h, depending mainly on the state of activity of the AS1 inoculum, which was mainly conditioned by the operation of the local treatment plant, but was on average equal to 4.659 and 16.80 mg/L·h for the inocula AS2 and AS3 respectively. This influenced the transfer of oxygen in the liquid phase which was controlled as previously mentioned by the operating conditions set in the respirometric tests and the inocula microbial activity. The AMX effect introduction on microbial activity could be manifested under different aspects either by slowing down the microbial activity and therefore increasing the time recommended for the degradation of the exogenous substrate injected into the respirometry reactor (T_s), or by a stopped activity of some microorganisms, reducing the exogenous heterotrophs maximum respiration, or by simultaneous effects and consequently a synchronized significant reduction in time and maximum respiration would be recorded. A multitude of experiments was performed, adding different quantities of AMX and the same concentration of synthetic substrate. Nevertheless inocula AS2 and AS3 were carefully washed before carrying out the respirometric tests not only to reach the endogenous state, but also in order to reduce the salinity of the reaction medium caused by the use of the substrate in salt form. Inhibitory effects of AMX on the three activated sludge activity are summarized in (Table 7), The parameters, defined by using a respirometer were used to obtain information about the effects on the two of the most important biochemical processes that take place in a wastewater treatment plant, heterotroph biomass growth (Y_H) and heterotroph energy substrate consumption ($Y_{O/S}$). As can be seen, the fraction of the substrate use for the energy consumption was very dissimilar for the different types of inocula, in general it was less weak for the AS1 and AS2 inoculum and more significant for the AS3 inoculum. The effect of the introduction of AMX on these different types of biomass was not very regular (Table 8).

Maximum reductions in the $Y_{O/S}$ rate expressing the fraction of the substrate used for the energy consumption were 30.639%, 21.79% and 19.923% for the 75, 120 and 150 mg/L AMX doses and inocula AS1, AS2 and AS3, respectively (Fig. 9) [74]. The effect of the AMX introduction seemed at times to slow down microbial activities since the recommended time (T_s) for the degradation of the injected substrate increased without attenuation of the maximum respiration in some experiments and at times reduced the activity since the maximum exogenous respiration and the degradation time decreased significantly, reaching 29.883%, 36.844% and 25.243% for the doses 75, 120 and 100 mg/L of AMX and the inocula AS1, AS2 and AS3, respectively.

According to Fig. 9 showing the percentages inhibition of (a) heterotrophs for the three different activated sludge used and (b) for the mixed liquor without nitrification inhibitor. The heterotrophs adapted to the substrate and substrate containing AMX showed remarkable resistance

Table 7
Inhibition percentage relative to DO consumed by heterotrophs for substrate injected

Dose (mg_{AMX}/L) inhibition%	5	15	30	50	60	75	100	120	150
AS1	2.167	–11.039	–19.067	17.467	–	30.646	0.775	–	–
AS2	–	–8.339	–0.764	–	12.380	–	–	21.746	–
AS3	18.039	16.017	9.229	–5.093	–	–1.362	15.244	–	19.934

Table 8
Estimated $Y_{O/S}$ before and after different doses of AMX injection

		5	15	30	50	60	75	100	120	150
AS1	$Y_{O/S}$	0.181	0.190	0.167	1.164	–	1.126	0.996	–	–
	$Y'_{O/S}$	0.177	0.211	0.199	0.960	–	0.781	0.988	–	–
AS2	$Y_{O/S}$	–	0.272	0.295	–	0.267	–	–	0.234	–
	$Y'_{O/S}$	–	0.295	0.297	–	0.233	–	–	0.183	–
AS3	$Y_{O/S}$	1.225	0.861	0.975	1.223	–	1.077	0.842	–	1.044
	$Y'_{O/S}$	1.004	0.723	0.885	1.286	–	1.091	0.714	–	0.836

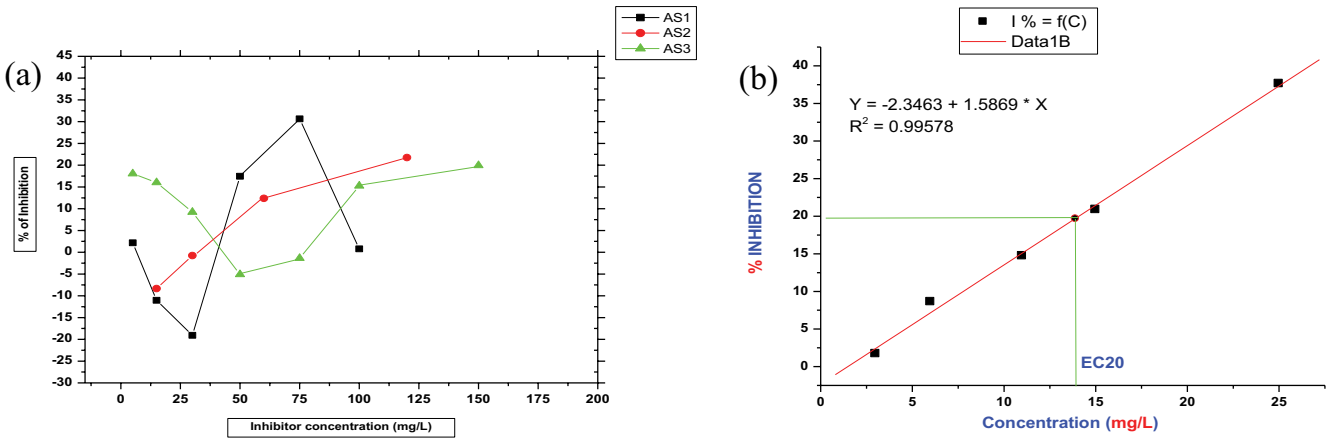

Fig. 9. Inhibition percentage curve according to inhibitor concentration for (a) the different heterotrophic biomasses (AS1, AS2, AS3) and (b) the mixed liquor (heterotrophic and autotrophic).

even at concentration up to 150 mg_{AMX}/L and that did not exceed 20% inhibition, the un-acclimated heterotrophs were inhibited at 35% for the 75 mg_{AMX}/L, which shows that heterotrophs were less sensitive to the inhibition and had a rapid adaptive capacity, since the mixed liquor, which contained the heterotrophs and autotrophs, was more sensitive and less adaptable. The autotrophs present in mixed liquor, exhibited no inhibition at 40 mg_{AMX}/L, whereas a 35% inhibition at 25 mg_{AMX}/L was shown, and this percentage was found for not adapted heterotrophs at 75 mg_{AMX}/L. Generally, AMX effect was really unexpected with no logarithmic function expressing the inhibition kinetics. The heterotrophs were more resistant to inhibition and adapt quickly to their removal. According to the literature, there is still a gap on AMX toxicity effect in nitrifying biomass. AMX can exhibit toxicity in nitrifying bacteria at concentrations ranging from 0.75 μg/L and their removal by biological processes is in the order of 80% from a 100 ng/L concentration in AS process. Complete nitrification inhibition has occurred at a 200 mg/L concentration, showing that the nitrifying bacteria were more sensitive to AMX or to many other micro-pollutants (such as heavy metals) than microorganisms responsible for the carbonaceous material oxidation (heterotrophs) [85]. This showed a great acclimation capacity and less sensitivity to different inhibitions [86–88] and is known to govern COD removal and is also suggested for PhACs and AMX removal at the presence of a high number of active heterotrophic biomass [89]. However, other research [90] showed that the AMX addition did not affect the COD and DOC (dissolved organic carbon) removal efficiency and did not inhibit the heterotrophs metabolism at certain rates.

4. Conclusion

The respirometry use in this study provides important immediate and direct information to protect aquatic ecosystems upstream and downstream of WWTP. The operating conditions of this technique are well defined in terms of the mixed liquor preparation by washing and the operating conditions imposed for K_La determination, as well as the initial ratio S_0/X_0 which makes it possible to reduce the experiments times and to make successive injections avoiding bacterial reproduction or growth. AMX has unexpected effects on the biomass of activated sludge, but heterotrophs show resistance capacity at fairly large AMX doses, especially in the presence of sodium acetate which improves the ability of microorganisms to resist the stress provided by the AMX. This study is part of the research to know the effects caused by emerging micropollutants on the purifying biomass of conventional WWTP in order to avoid malfunctions, and allows exploiting these results with previous results to identify and develop an elimination pathway by natural and inexpensive methods based on the respirometric technique or coupling of processes.

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