Experimental evaluation of pharmaceuticalproducts effect on activated sludge activity:Case of Amoxicillin

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Abstract

The presence of biologically resistant substances, such as antibiotics which are considered inhibitors, in wastewater deteriorates biological treatment systems (activated sludge) by reducing microbial activity which in turn impacts negatively carbonaceous treatment, solid- liquid separation etc. In this work, activated sludge under different conditions were used to evaluate experimentally the Amoxicillin effect and they were as follows: not adapted biomasses of conventional WWTP, adapted biomasses to the binary substrate (acetate and ammonium), adapted biomasses to the Amoxicillin and binary substrate, finely the biomasses adapted to another organic substrate (other than acetate). The experiments were undertaken using continuous aeration respirometric technique. This is based on monitoring oxygen uptake rate, of samples containing Amoxicillin and others free of it, as well as soluble COD and Amoxicillin, consumption kinetics using UV-VISIBLE absorbance measurements.

The inhibition level was evaluated using the effective concentration index (EC index). Two ranges of Amoxicillin concentrations of 3 mg/l to 26 mg/l and 5 mg/l to 150 mg/l were used. Inhibition starts to appear at a low concentration of less than 3 mg/l in non adapted biomasses and in activated sludge adapted to organic substrate other than acetate, EC attains 14.71 % at a concentration of 11.5 mg/l for, however, a low S_0/X_0 ratio. Furthermore, the adapted activated sludge appeared to resist to Amoxicillin with a high concentration. It should be underlined that the results can be considered as preliminary, because, the antibiotic Amoxicillin trihydrate remains a very complex molecule and represents a different stress effect on the purifying bacteria. Further work is needed in order to evaluate the exact concentration in the low concentration range, and to determine the resistant germs through their insulation as well as the application of these protocols to higher S_0/X_0 ratios.

***Keywords**: Activated Sludge, Adaptation, Amoxicillin, Inhibition, Respirometry.*

I. Introduction

Currently, the domestic wastewater processing is carried out in wastewater treatment plants (*WWTP*) based, for the majority,on biological process of activated sludge. The presence of various types of natural occurring bacteria (heterotrophic and autotrophic) in the aeration tank insures organic matter degradation (*C, N* and *P*)[03] accompanied with its growth. However, problems concerning the presence of micropolluants, toxic and resistant to biodegradation, can exert an inhibiting effect on the activated sludge[04].Consequently, the effectiveness of biological treatment[05-07]of the effluent deteriorates significantly even at low concentrations (*µg/L, ng/L*).

Among the micropolluants present in *WWTP*, reported in several studies[08-13], antibiotics and

active pharmacological products [14-16] used in veterinaryand human medicine[17-18].The world consumption of antibiotics is estimatedbetween *100.000and 2.000.00 t/year*, with β-lactam accounting for *50-70%* of the total antibiotic usage[19].TrihydrateAmoxicillin is a β-lactam antibiotic.It is considered as the drug of choice because of its better absorptive characteristics after oral administration.The physicochemical properties and chemical structure of Amoxicillinare represented in ***Table 01***and ***Figure 02*** respectively.The perfect tool to evaluate toxicity (or inhibition) in *WWTP* does not exist[20]yet. However, the respirometric method is largely used in the assessment of the inhibiting effect of Amoxicillin on the microbial activity because of its simplicity, speed and profitability[21-22]. This method is designed on the basis of standardized method of inhibition tests (*ISO-8192*). It is based on activated sludge oxygen uptake rate (*OUR*) measurements[05]which decreases when wastewater contains the inhibitor[24-25]. A significant parameter in the assessment of inhibition is the *EC20* value defined as the effective concentration of the inhibitor inducing *20%* reduction of oxygen uptake rate[05].Details on the pharmaceutical product used in this study (Amoxicillin), the respirometric setup and the different protocolsused are detailed in the materials and methods.

In this context, the experimental procedure employed in this work to analyze the inhibition of biomass enables us to determine to a certain extent the instantaneous effect of Amoxicillin and do not consider the potential adaptability of the biomasses after a proportioned time of acclimatization. The obtained results provide a preliminary data on the inhibiting effect of Amoxicillin on activated sludge. An increase in the inhibitor concentration resulted in a partial or total weakening of the performance of the biological system.

II. MATERIALSANDMETHODS

The respirometer:

The assembled respirometer used in the experimental work of this study, is an open and aerated reactor, developed in a preceding work [44]and does not require any reagent or complex apparatus during the test.In this installation which contains a reactor made of glass of *0.5 L* (**04**), the air is supplied by an aquarium pump of *SHARK-RS-510* (**02**), which delivers a maximum air flow of *150 L O_2/h* approximately through plastic diffuser.The liquid phase was mixed in the batch reactor using a bar magnet and a magnetic stirrer *AGIMATIC-N* (**05**).The *pH, DO* and *temperature* were measured in liquid phase using respectively a pH probe *IDS-WTW* (**07**), an oxygen probe *FDO-925 IDS* of *WTW* (**03**), which were connected to a *Multi-Oxymeter -3430 of WTW* (**08**) and a thermometer.The system was maintained at a constant temperature of *20 °C ± 0.5 °C* (**01**) in a thermostatic enclosure of *WTW* (**06**).***Figure 01***shows a picture of the experimental setup used inrespirometric tests.

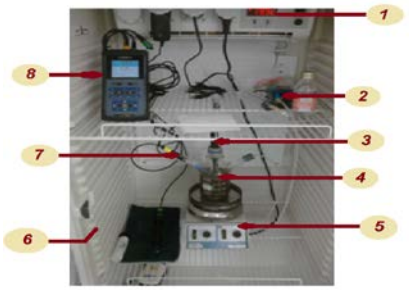

Fig. 01:Picture of the respirometric setup used in this study.

Chemicals:

TrihydratéAmoxicillin:

Amoxicillin prevents the synthesis of bacterium cellular wall, they prevent the reticulation between the linear chains peptidoglycane of polymer which is a significant component in the composition of the cellular wall.Indeed, Amoxicillin, while being fixed on proteins of penicillin connections (*PLP*), present at the surface of the cytoplasmic membrane of the bacteria, therefore inhibit the enzymatic activity of the *PLP*, enzyme necessary to the assembly of peptidoglycane.The fixing of Amoxicillin with *PLP* entrained the stop of partial synthesis and this fact inhibits the bacterial growth due to the embrittlement of the wall which leads then to bacterial lyses[45-47].Amoxicillin in this study was selected on the basis of several criterions, among them, their raised consumption, their effect on environment and microorganisms.In order to evaluate the impact of Amoxicillin on the activated sludge wastewater treatment operation, various experimental procedures were proposed in the literature [49-50].

Tab. 01:Amoxicillinphysicochemicalproperties ofAmoxicillin. [19, 47, 51-52].

IUPAC(1) Name	α-amino-hydroxybenzylpenicillin
Synonyms	Amox ; AMC ; Amoxicillin trihydrate ; Amoxicillin anhydrous ; DAmoxicillin ; p-Hydroxyampicillin
Molecular formula	Amoxicillin: $C_{16}H_{19}N_3O_5S$; Amoxicillin trihydrate: $C_{16}H_{19}N_3O_5S*3H_2O$
No. CASRN(2)	26787-78-0
Molecular Weight	Amoxicillin: 365.40 gm/mol; Amoxicillin trihydrate: 419.41 gm/mol.
Molecular Width	1.32 nm
Physical characteristics	Solid or liquid, white to off-white crystalline powder, Penicillin-type odor, slightly soluble out water and alcohol such as ethanol-methanol
Solubility in water	3430 mg/L water
Melting point	194 °C
Boiling point	743.2 °C at 760 mmHg
Flash point	403.3 °C

pK_a[3]	3.39, 6.71, 9.41
log KOW[4]	0.87

(1). International Union of Pure and Applied Chemistry;
(2). Chemical Abstract Services Registry Number;
(3). Acid dissociation constants;
(4). Octanol/water partition coefficient.

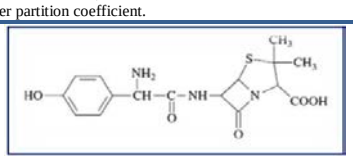

Fig. 02: Amoxicillin structure [19].

Synthetic effluent (binary substrate):

In order to ensure constancy in wastewater quality during the different tests, an easily biodegradable binary synthetic effluent was used in this work. It comprises a carbon source, a nitrogen source and micronutrients. Mixed in distilled water, the solution *pH*was adjusted to a *7.5* value using either *HCL* or *NaOH*.The synthetic wastewater composition is represented below in ***Table 02***[53-54].

Tab. 02: Composition ofthe syntheticwastewater solutionused in the respirometric tests[55].

Constituents	Concentration (mg/L)
Carbonsource:$C_2H_3O_2Na$	2.570
Nitrogensource:NH_4Cl	1.485
Les nutrients:	
NH_4Cl-N	6.8
$FeCl_3$	2.3
$MgSO_4$	166
$CaCl_2$	412
Phosphate Plug: pH, 7.24	
K_2HPO_4	410
KH_2PO_4	90

The synthetic wastewater prepared with a theoretical *COD* concentration of *2000 mg/l* was stored at a temperature of *4 °C*.

Sources and preparation of activated sludge:

The biomass used in the different respirometric tests originated from *IBN ZIAD* municipal *WWTP* which treats Constantine wastewaters. It is designed with treatment capacity of *800 l/s* (*450 000 Eq-inhabitants*). The sludge samples were collected manually from the aeration basin, transported to the laboratory in shaded glass bottles at a temperature of *4°C*. To prepare the samples in the laboratory prior to respirometric testing, a *400 ml*of the activated sludge were washed thoroughly with distilled water then maintained under continuous aeration during *3 hours* maximum at a temperature of *20 °C* to be sure that all external substrate has been consumed and the endogenous respiratory is the only process.

The sludge washing process can remove *85* to *95 %* of the initial substrate concentration; ***Figure 03***gives additional information on the reduction effectiveness and percentages of this technique obtained on sludge samples of a substrate/biomass ratio $S_0/X_0 = 0.04$.

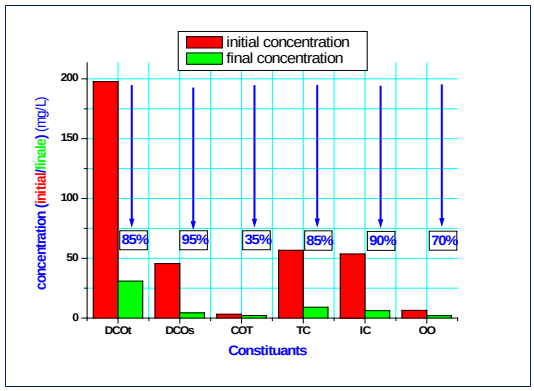

Fig. 03: histogram represents the reduction percentages of substrate concentration of activated sludge after washing.

Toxicity evaluation:

Generally, the effect of an inhibitor on activated sludge system is usually expressed like a repressive action on the growth of the microorganisms.Various models of inhibition were developed up to now, the methods of respiration *ISO-8192* (*1986*) and *OECD-L133/118* (*1988*) based on breathing, describe the inhibiting effects of a substance on the activated sludge by measuring the respiratory rate under defined conditions in the presence of an easily biodegradable substrate like acetate and different concentrations of thetoxic elements.

A significant parameter in this method is the value of *%* inhibition defining the effective concentration of inhibitor inducing a reduction of oxygen uptakerate:

$$\%\ Inhibition = \left(1 - \frac{OUR_{inhibited}}{OUR_{control}}\right) * 100 \qquad (02)$$

The respirometric tests for the detection of inhibition is very useful since the results are obtained quickly, but for a more quantitative description of the toxic effect it is preferably employed in combination with the measurements of *EC20*value. The effective concentration, even for *EC50* and *EC80*, which representrespectively the concentration of a compound where*20 %*, *50%* and *80%* of its maximum effect are observed.This value was derived by a linear interpolation on the plot of the curve of inhibition percentage versus concentration of the inhibitor by ***Equation 03***:

$$EC20 = \frac{OUR_{control} - OUR_{inhibited}}{OUR_{control}} * 100 = 20\ \% \qquad (03)$$

Experimental procedure:

Examined inhibition with the respirometric test should simulate the true inhibition in the *WWTP*. To check this, several methods are cited in the literature, which consistedof comparing *OURs* obtained through a successive injection of synthetic effluent without inhibitor followed by the injection of the synthetic effluent with the inhibitor,after the return to the endogenous state (depletion of the external substrate). The experiment is repeated for different concentrations of the inhibitor.

Analyzed parameters:

The characterization of the activated sludge was carried out by the determination of *TSS*, *VSS*, *COD*, BOD_5, V_{30}, *SVI*, *TOC*, *TC* and *IC*according to standards methods[56-57].***Table 03***shows the activated sludge characterization for the different collected samples for all this study.

Tab. 03: Characteristics of activated sludge used during the probation period.

Paramètres	Concentration*
TSS (mg/L)	6700 ± 1605
VSS (mg/L)	2992 ± 618
VSS/TSS	0.447 ± 0.38
V_{30} (ml/L)	192 ± 27.5
SVI (ml/g)	0.029 ± 0.017
pH	7.419 ± 0.106
DO (mg/L)	4.22 ± 0.09
COD_t (mg/L)	197.61 ± 22.58
COD_s (mg/L)	40.28 ± 8.97
BOD_5 (mg/L)	395 ± 15
TOC (mg/L)	3.745 ± 0.595
TC (mg/L)	56.89 ± 0.24
IC (mg/L)	53.135 ± 0.365
BOD_5 /COD	1.999 ± 0.664
COD/BOD_5	0.500 ± 1.50
COD/ TOC	52.77 ± 37.95
BOD/TOC	105.47 ± 25.21
Conductivity	1408.5 ± 55.5
Salinity	0.65 ± 0.05

*: results concern all collected samples used in this study

III. RESULTS AND DISCUSSION

Oxygen Uptake Rate

The oxygen uptake rate *OUR*,was determined by measurements ofdissolved oxygen concentration variation inthe reactor, when the aeration is stopped, according ***Equation 04***:

$$OUR = -\frac{dS_O(t)}{dt} \qquad (04)$$

Determination of K_La and OUR_{end}

Equation 05 describes the variation of dissolved oxygen concentration at the endogenous state under continuous aeration, whereas ***Equation 06***describes this variation under no aeration with endogenous respiration only. Consequently, the slope of the linear portion of the descending curve is equal to *OURend*.

$$\frac{dS_O}{dt} = K_La * (S_O^* - S_{end}) - OUR_{end} = 0 \qquad (05)$$

$$\frac{dS_O}{dt} = -OUR_{end} = -\alpha \qquad (06)$$

With α, the downward line slope of dissolved oxygen versus to time;
Therefore, OUR_{end} in ***Equation 05*** can be replaced with its value "α" and knowing that S_O is constant ($dS_O/dt = 0$) therefore K_La can be calculated with ***Equation 07***. ***Table 04*** shows the calculated$K_La$values during the inhibition tests.

$$K_La = -\frac{\alpha}{(S_O^* - S_{end})} \qquad (07)$$

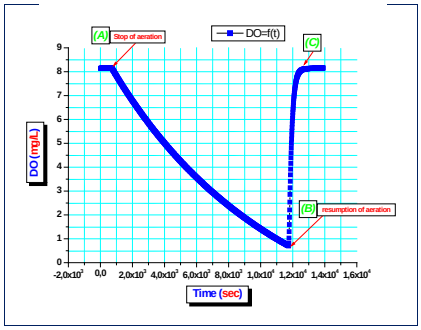

Fig. 04:an example of *DO*concentration variation curve versus time during the phase of stop/resumeaeration under endogenous conditions.

Respirogrammes obtained after various injections

Figure 05 represents a respirogramme describing the endogenous respiration with external aeration at the beginning of the experiment (*at A*), then an endogenous respiration without external aeration showing the descending curve (*A-B*), this part of the curve will be used to calculate the volumetric oxygen transfer coefficient K_La. After resuming aeration (*B*) the curve returned to the endogenous respiration state with external aeration (*C*), the level of oxygen in the solution is similar to the initial state (*C-D*). After injecting the binary substrate, carbonaceous and nitrogen sources, (*D*), with maintaining external aeration, the *DO* concentration diminishes, due to its consumption by the bacteria. In fact the first portion of this part represents the carbonaceous consumption (*D-E*) and the second part represents the nitrogen consumption (*E-F-G*). To differentiate between the two oxidation processes, the value of the *pH* can be used as an indicator. In fact, from point (*E*) to (*F*)

the *pH* is lower than from (*D-E*) indicating that there is an acidity production due to nitrification Moreover, the consumption kinetics of Oxygen during nitrification is lower than the carbonaceous one.

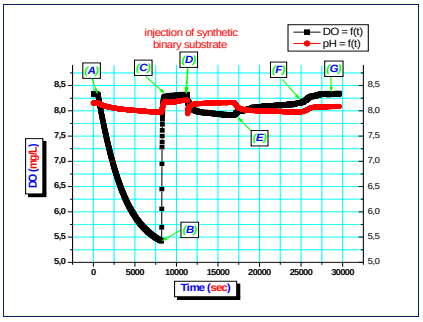

Fig. 05:example of a respirogramme of *DO* and *pH*versus time after the injection of synthetic effluent.

Consecutive injection of the binary substrate without and with antibiotics

Figure 06 represents a consecutive injection of a binary substrate, showing the first injection, the return to the endogenous respiration then the second injection. The form of *DO* variation for both injections is similar. ***Figure 7*** and ***Figure 8*** show respectively a superposition of *DO* variation and *OUR* variation curves of both injections, indicating that the oxygen consumption of the second injection is different with a *15%* variation in the consumption.

Figure 9 represents a respirogramme of consecutive injections of the binary substrate. The first injection consisted of the substrate free of any antibiotics whereas the second one with the antibiotics. Similarly ***Figure 10*** and ***Figure 11*** shows respectively a superposition of *DO* variation and *OUR* variation curves of both injections, indicating that the oxygen consumption in the presence of the antibiotic is low representing *20%* of the consumption without antibiotics therefore a reduction of *80%* resulted.

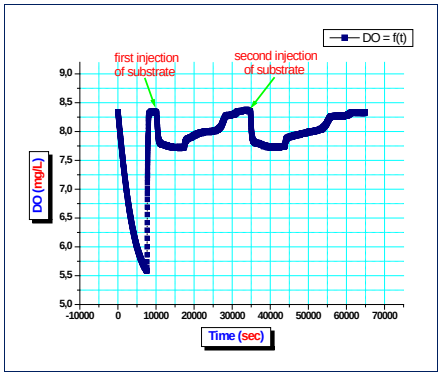

Fig. 06:example of a respirogramme obtained after successive injection of same substrate without inhibitor.

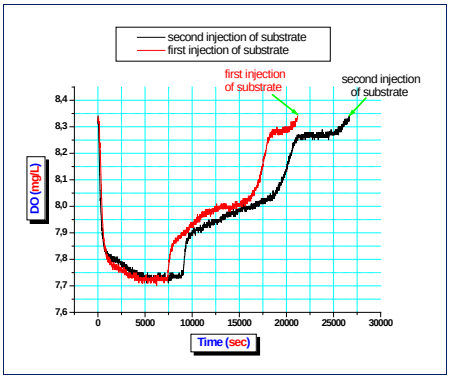

Fig. 07:example of a respirogramme of *DO* concentration versus time for two successive injections of same substratewithout inhibitor.

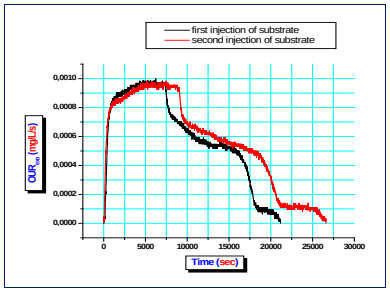

Fig. 08:example of a respirogramme of *OURexo* versus time obtained after integration of *DO* curve for successive injection of same substrate.

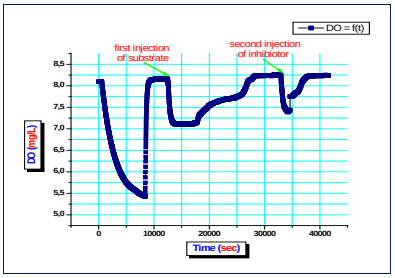

Fig. 09:example of a respirogramme of successive injection of binary substrate followed by injection of binary substrate containing certain concentration in Amoxicillin.

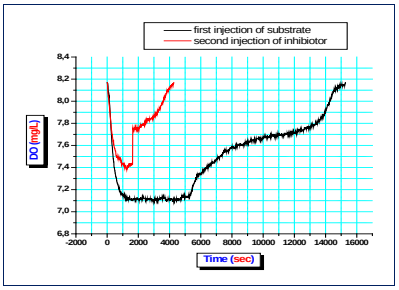

Fig. 10:example of a respirogramme obtained after successive injection of substrate and that of substrate containing the inhibitor.

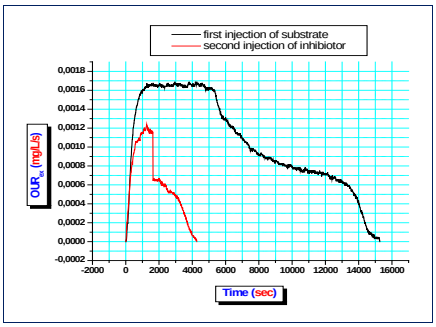

Fig. 11: The OUR,versus time for the successive injection of substrate free of Amoxicillinfollowed by the substrate containing the antibiotic.

Tab. 04:Calculated OUR, from ***Figure 8*** and ***Figure11***.

OURex	Area
OURex S1	12,69019
OURex S2	15,04099
OURex S1	15,8961
OURex S+I	2,66437

Inhibition variation with the various antibiotics concentrations

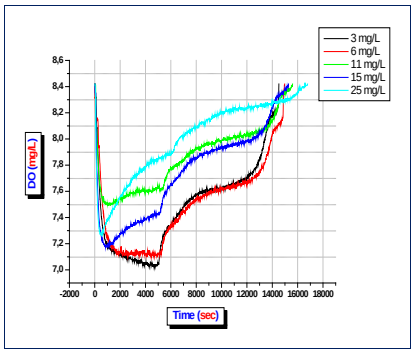

Fig. 12:respirogramme of the various injections testing of substrates only.

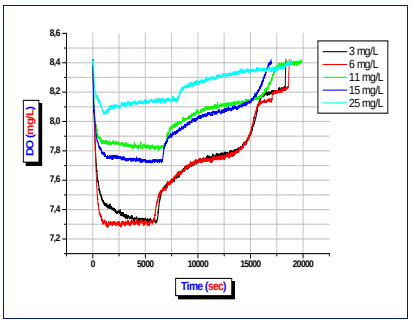

Fig. 14:respirogramme of the injections of various substrates containing the different concentrations of Amoxicillin.

In order to assess the variation of the biomass activity inhibition due to the presence of antibiotics, different concentrations *3*, *6*, *11*, *15* and *25 mg/l* were tested. The results of *DO* and *OUR* variations for the dual substrate alone are represented respectively in ***Figure 12*** and ***Figure 13***. The variations in the results are due to the fact that the biomass has not been acclimated and the collect date of activated sludge from the *WWTP* is different as well. Moreover, ***Figures 14*** and ***Figure 15*** represent respectively the results of *DO* and *OUR* variations of the dual substrate with the presence of the antibiotics. To evaluate the level of inhibition, the *OUR* for substrate alone is calculated from ***Figure 13*** and compared to the *OUR* of the substrate mixed with the antibiotics, calculated from ***Figure 15***. The overall results are recapitulated in ***Table 5***, with the calculation of the *EC index*, and the *OUR* results are represented in ***Figure 16***.

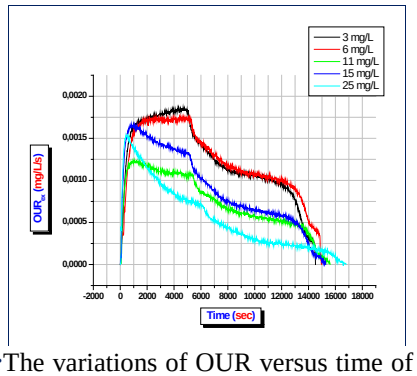

Fig. 13:The variations of OUR versus time of various injections tests of substrates only.

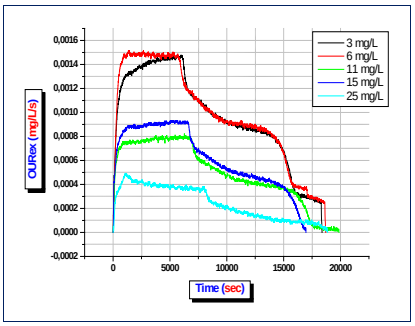

Fig. 15:*OUR* consumption versus time of the injections of various substrates tests containing the different concentrations of Amoxicillin.

Tab. 05:table shows the results obtained in the experiments of inhibition.

COD_{eff} synthetic	S_0/X_0	S_0/X_0 Reality*	[Inhib] (mg/L)	% inhibition	V_{eff} mL	V_b mL	OUR_{end}** (S et S+I)	K_La (s^{-1})	OUR_{exo} S***	OUR_{exo} S + I****
300	0,04	0,039	3	1.7	120	280	0,00107	0,00137	11,1192	10,9309
300	0,04	0,041	5.5	8.61	110	290	0,00107	0,00115	11,5845	10,5874
300	0,04	0,039	11.5	14.71	115	285	0,00091	0,00133	11,4340	9,7519
300	0,04	0,039	16	20.86	115	285	0,00104	0,00160	17,2752	13,6712
300	0,04	0,039	26	37.6	105	295	0,00071	0,00120	10,0645	6,2767

*: real value of S_0/X_0 in reactor
**: value of O*URend*obtained in curves of the successive injection of substrate only (*S*) and substrate with inhibitor (*S+I*).
***: value of O*URexo*calculated for curves after the injection of substrate only (*S*).
****: value of O*URexo* calculated for curves of the injection of substrate with inhibitor (*S+I*).

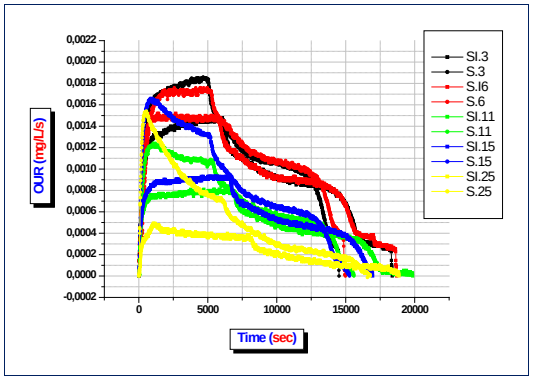

Fig. 16:variation of *OUR* consumption versus time of the different samples of binary substrate without and with different concentrations of Amoxicillin.

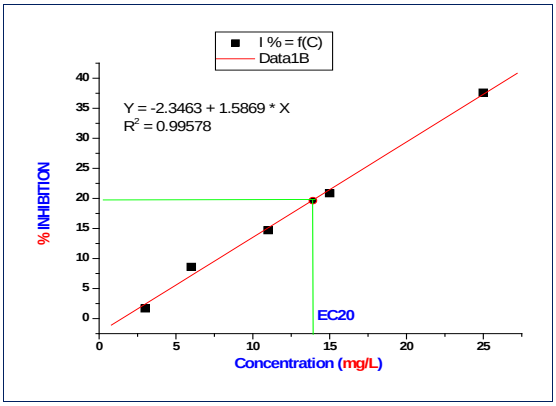

Fig. 17:curve of percentage of inhibition according to concentration in inhibitor for the evaluation of *EC20*.

Figure 16 shows clearly the effect of inhibition on oxygen consumption where an overall reduction of oxygen consumption can be noticed on the curves, indicating a reduction of biomass activity. This can be seen on the curves corresponding to *25*, *15* and *11 mg/l* of Amoxicillin, where biomass activity has been inhibited almost completely resulting in a lowering of the maximum *OUR* (plateau), contrarily the residence time necessary to consume the total binary substrate is increased. Furthermore the presence of the inhibitor did not alter the form of the *OUR* curves, indicating probably that the processes in presence did not change in nature but have changed in their celerity.

Data from ***Table 5*** concerning inhibition Percentage and inhibition concentration were plotted in ***Figure 17*** in orders to interpolate the *EC20* value. The obtained curve has a good linear form in the studied interval.

The study shows the inhibiting effect of Amoxicillin on a synthetic binary substrate effluentbiodegradation. At a concentration of *3 mg/l* of Amoxicillin, inhibition starts to appear (*1.7%*) and increased thereafter to attain *37.6%* for an Amoxicillin concentration of *26 mg/l*. It should be underline that the antibiotics concentrations in wastewater effluents vary considerably, from *720µg/l* in a hospital effluent to a level of *31mg/l* in an industrial one[63]. Furthermore ***Table 5*** shows a decrease of *OUR* for the different Amoxicillin concentrations which is equal to the respective values of inhibition percent.

IV. CONCLUSION

The discharge of antibiotics and their residues can disturb the watery ecosystems[64-65], and bacterial activity in nature or in wastewater treatment plant. The majority of the *WWTP* are not designed to face the antibiotics which can be toxic to the biomass in aeration basin.The presence of Amoxicillin has been found to start to inhibit bacterial activity from a concentration less than *3 mg/l* and attain *37.6%* for a concentration of *26 mg/l*. Moreover, inhibition is expressed through a reduction in the celerity of the kinetics of the processes present but not by changing their nature.

The *EC20* of the studied Amoxicillin was found to correspond to a concentration of *14.53 mg/l*.

This study has led us to apply the *EC* determination procedure, which is quite easy to implement in a laboratory if respirometric equipment are available. Moreover, it would be interesting to assess the influence of Amoxicillin at low concentration and to verify the removal process is of a biological nature alone.

Nomenclature:

EC: (%) effective concentration index.
S_0/X_0: (mg_{COD}/mg_{VSS}) initial substrate-to-biomass ratio.
OUR: (*mg/l/h*) Oxygen Uptake Rate
OUR_{end}: (*mg/l/h*) Endogenous Oxygen Uptake Rate
OUR_{exo}: (*mg/l/h*) Exogenous Oxygen Uptake Rate.
$OUR_{CONTROL}$: (*mg/l/h*) the maximum specific respiratory rate detected before the addition of toxicant.
$OUR_{inhibited}$: (*mg/l/h*) the maximum specific respiratory rate detected after the addition of toxicant.
K_La: (h^{-1}) Oxygen Transfer Coefficient
S_O: (*mg/l*) dissolved oxygen concentration.
S_O*: (*mg/l*) dissolved oxygen concentration in saturation.
COD: (*mg/l*) chemical oxygen demand.
COD_S: (*mg/l*) soluble chemical oxygen demand.
CODt: (*mg/l*) total chemical oxygen demand.
COD_{eff}: (*mg/l*) chemical oxygen demand of synthetic effluent.
TSS: *(mg/l*) total suspended solid.
VSS: (*mg/l*) volatile suspended solid
BOD_5: (*mg/l*) biological oxygen demand after 5 days
V_{30}: (*ml*) elutriated volume of sludge after 30 minutes.
SVI: (*ml/l*) Sludge volume index
TOC: (*mg/l*) total organic carbon
TC: (*mg/l*) total carbon
IC: (*mg/l*) inorganic carbon
V_{eff}: (*ml*) volume of effluent injected in reactor
V_b: (*ml*) sludge volume in reactor
α: downward line slope.
WWTP: wastewater treatment plant.
C, N, P: carbon, nitrogen and phosphor
PLP: proteins of penicillin
DO: dissolved oxygen.

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