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Original Article

Assessment of toxicity and kinetic effects of erythromycin on activated sludge consortium by fast respirometry method

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Abstract

Background: The present study aimed to assess the acute impact of erythromycin (ERY) as an inhibitor on peptone mixture utilization of activated sludge (AS) consortium.

Methods: For this purpose, the inhibition of oxygen consumption was used based on the ISO 8192:2007 procedure. In this method, the AS consortium (10-day age) was extracted from lab-scale membrane bioreactor, then, percentage inhibition for total, heterotrophic, and nitrifying microorganisms, in separate batch respirometric tests were calculated in the absence and presence of N-allylthiourea (ATU) as a specific Nitrification inhibitor.

Results: The obtained data showed that the height of oxygen uptake rate (OUR) profiles and amount of oxygen consumption reduced with increasing ERY dose. The half-maximal effective concentration (EC_{50}) of ERY for heterotrophic and nitrifier microorganisms were 269.4 and 1243.1 mg/L, respectively. In Run 1, the kinetic coefficients b_H, $f_{A,H}$, Y_H, and μ_H were calculated as 2.61 d^{-1}, 0.44, 0.4945 mg VSS/mg COD, and 0.047 d^{-1}, respectively. Also, for maximum ERY concentration (1000 mg/L), the kinetic coefficients b_H, $f_{A,H}$, Y_H, and μ_H were calculated as 2.27 d^{-1}, 0.3, 0.4983 mg VSS/mg COD, and 0.0049 d^{-1}, respectively.

Conclusion: The findings showed that the inhibitory impact of ERY was observed as a decrease in the amount of oxygen consumption by OUR profiles in rapid respirometric method (ISO 8192), which offered a novel insight for the acute inhibitory impact of this antibiotic. Also, chemical oxygen demand (COD) as an overall substrate parameter is most helpful in interpreting the behavior and the metabolic functions of AS systems.

Keywords: Toxicity, Erythromycin, Activated sludge, Respirometry, Kinetic coefficients

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Introduction

Antibiotics are one of the most important groups of pharmaceutical active compounds that have been widely used in medicine and veterinary medicine (1,2). Unfortunately, the increasing production and use of these compounds have led to their release into the environment and pollution of surface water, groundwater, and soil (3). A large part of these compounds is excreted unchanged or as metabolites, which after disposal in the municipal wastewater system, enter the aquatic environment and groundwater aquifers (4). It should be noted that in most cases, wastewater treatment plants (WWTPs) are inadequate to remove the antibiotics discharged in them,

which eventually enter the aquifer with effluent (5). So, they may have the inhibitory or toxicity potential to the biomass in biological treatment systems, affecting their performance, and also, increasing bacterial resistance crisis (6,7).

Among antibiotic compounds, the macrolide group, including erythromycin (ERY), is one of the most extensively used drug compounds worldwide (8).

This compound has a molecular weight of 734 and a solubility of 30 g/L and log coefficients of Kow and PKa are equal to 0.28 and 6.09, respectively (9,10).

ERY as a macrolide used in both human and veterinary medicine, inhibit protein biosynthesis and can be used to

treat infections with a wide variety of gram-negative and gram-positive bacteria (7,8). Recently, researchers have reported that the occurrence of macrolides, specially ERY, in environmental samples with concentrations ranging from ng/L to a few µg/L in various global water bodies, but their fate and effect on WWTPs have been the subject of limited studies (11-14).

Louvet et al reported that ERY affected the specific nitrification rate and the structure of activated sludge (AS) flocs from different WWTPs (15). Pala-Ozkok and Orhon reported that ERY has a toxic effect on the microbial community and resulted in an inactivated fraction of biomass (16). Also, in other researches, they reported that ERY affected substrate storage by accelerating the endogenous respiration to prevent the toxic effects of ERY (7,16,17).

There are different techniques implied for assessing the antibiotics toxicity impacts, especially on substrate utilization and aerobic microbial consortium (AMC) in the biological wastewater treatment process (18).

To date, the pharmacological toxicity of various pharmaceuticals has been determined using the Microtox assay of bioluminescence experiments with *Vibrio fischeri* (19,20). Furthermore, Onesios and Bouwer, based on the biofilm protein measurements in column searches, recommended pharmaceuticals toxicity to the biofilm populations (21). Newly, Ortiz de García et al examined the ecotoxicity of pharmaceuticals in aquatic environments and WWTPs using bioluminescence and respirometry tests, respectively (22). The AS respirometric experiment is supposed as a primary approach for evaluating the toxic influence of substances (e.g. pharmaceuticals) on sludge activity and its potential effect on its microbial community (18).

The respiratory analysis is a sensible and cost-effective approach based on oxygen consumption via biological AS (22). Hence, respiration has been widely used to define the possible toxicity of various distinct chemical compounds in the microbial community of WWTPs sludge.

The International Organization for Standardization (ISO) is a worldwide federation of national standards bodies. The ISO 8192 method as a respirometry technique is a method for assessing the inhibitory effect of a test material on the oxygen consumption of AS microorganisms.

This method is intended to represent the conditions in biological WWTPs. It gives information on inhibitory or stimulatory effects after a short exposure (usually 30 minutes up to 180 minutes or even more) of the test material on AS microorganisms.

The information generated by this method for assessing the potential toxicity of substances, mixtures and, wastewaters to AS may be helpful in estimating the effect of test material on mixed bacterial communities in the aquatic environment, as separately. The susceptibility

of oxygen uptake by different sub-populations of the bacterial communities to inhibition by chemicals and wastewaters is not necessarily the same, and selective effects may profoundly influence the outcome of the test (23).

Thus, in the present study, the AS respirometry test (ISO 8192:2007 procedure) was used as the main experimental tool to assess sludge activity, toxicity, and substrate biodegradation affected ERY as an inhibitor compound by measuring the rate of oxygen consumption defined as oxygen uptake rate (OUR) profiles. These profiles are biodegradation fingerprints for any aerobic process under different operating conditions, and different parts of any one exhibited variable sensitivity for different model coefficients.

Materials and Methods
Chemicals and reagents
In the present study, ERY (Aldrich 637625, CAS-No: 114-07-8) was obtained from Sigma Aldrich Chemical Company (USA). Other chemicals including peptone from meat (Aldrich CAS No. 70175), meat extract (Aldrich), urea (Merck CAS-No: 57-13-6), sodium chloride (NaCl, CAS No. 7647-14-5), calcium chloride ($CaCl_2•2H_2O$, CAS-No: 10035-04-8) magnesium sulfate ($MgSO_4•7H_2O$, CAS-No: 10034-99-8), di-potassium hydrogen phosphate (K_2HPO_4, CAS-No: 7758-11-4), and N-allylthiourea (ATU) (CAS-No: 109-57-9) were purchased from Merck Co. (Germany). All chemicals are of analytical grade and used without further treatment. All working solutions were papered with the dissolution of the above-mentioned chemicals in deionized water.

Microbial consortium
AMC was extracted from a lab-scale membrane bioreactor and employed as a microbial seed for the toxicity experiment. The membrane bioreactor was fed with synthetic wastewater containing 2000 mg/L chemical oxygen demand (COD) at solid retention time of 10 days and dissolved oxygen (DO) concentration as near as possible saturation. The aerobic sludge was collected from the aerobic tank washed several times with deionized water, centrifuged, and re-suspended into deionized water to obtain the suspended solids (SS) of 3000 mg/L, and used as AMC in toxicity tests.

Batch toxicity assessment
In this study, the fast respirometry method was employed to assess the ERY toxicity according to the ISO 8192:2007 procedure (23). Due to well-balanced complex substrate and similarity of its composition to municipal and industrial wastewater, peptone and meat extract mixture was used as a sole substrate at a COD of 50 000 mg/L (24). The inorganic nutrients (listed g per liter of deionized water) were composed of urea (3 g), NaCl (0.7 g),

CaCl$_2$•2H$_2$O (0.4 g), MgSO$_4$•7H$_2$O (0.2 g), and K$_2$HPO$_4$ (2.8 g). After preparation of the AMC and synthetic ERY solution with the initial concentration of 1 to 1000 mg/L, the mentioned substrate was added to sealed batch reactors with the working volume of 500 mL, and the concentration of DO was measured at 15-minute intervals by a handheld Dissolved Oxygen meter (model 8403). At the beginning of the experiments, the amount of SS and DO in each batch test was fixed to 3000 and 3-6 mg/L, respectively.

In order to assess the ERY inhibition on total, heterotrophic, and nitrifying microorganisms, two sets of experiments were carried out in the absence and presence of N-allylthiourea (ATU) as a specific nitrification inhibitor. All experiments were conducted at 20 ± 2°C and pH = 7.5 ± 0.5.

Calculations

At first, the measurement data of the time and the DO values were taken. Then, the oxygen consumption rate (R), in milligrams per liter per hour, was calculated from the linear part of the recorded oxygen decrease graph according to Eq. (1):

$$R = ((\rho_1 - \rho_2)/\Delta t) \times 60 \qquad (1)$$

Where ρ_1 and ρ_2 (mg/L) are the oxygen concentration at the beginning and end of the selected section of the linear phase, respectively. Δt (min) is the time interval between these two measurements.

Also, the specific respiration rate (R_S) is expressed as the amount of oxygen consumed per dry weight of sludge per hour according to Eq. (2):

$$R_S = R/\rho_{SS} \qquad (2)$$

Where ρ_{SS} (g/L) is the concentration of SS in the test mixture.

The different indices of R (R_N, R_T, R_H) for blank values (R_{NB}, R_{TB}, R_{HB}), abiotic controls (R_{NA}, R_{TA}, R_{HA}), and assays with test material (R_{NS}, R_{TS}, R_{HS}) (mg/g·h), is calculated according to Eqs. (3) to (6):

$$R_N = R_T - R_H \qquad (3)$$

Where R_N (mg/L.h) is the rate of oxygen uptake due to nitrification.

R_T (mg/L.h) is the measured rate of oxygen uptake by the blank control (no ATU) (F_B).

R_H (mg/L.h) is the measured rate of oxygen uptake of the blank control with added ATU (F_N).

This coherence is valid for blank values (R_{NB}, R_{TB}, R_{HB}), abiotic controls (R_{NA}, R_{TA}, R_{HA}), and assays with test material (R_{NS}, R_{TS}, R_{HS}) (mg/g·h), which are calculated using Eqs. (4) to (6):

$$R_{NS} = R_N/\rho_{SS} \qquad (4)$$
$$R_{TS} = R_T /\rho_{SS} \qquad (5)$$
$$R_{HS} = R_H /\rho_{SS} \qquad (6)$$

In the same way, the oxygen uptake at different ERY concentrations (0-1000 mg/L) was determined.

Also, the percentage inhibition (I), for total, heterotrophic, and due to nitrification oxygen consumption at each concentration of ERY was calculated using Eqs. (7) to (9):

$$I = [1 - (R_T - R_{TA})/R_{TB}] \times 100\% \qquad (7)$$
$$I_H = [1- (R_H - R_{HA})/R_{HB}] \times 100\% \qquad (8)$$
$$I_N = [1 - (R_T - R_H)/(R_{TB} - R_{HB})] \times 100\% \qquad (9)$$

Finally, the percentage inhibition of oxygen consumption for total, heterotrophic, and nitrifying microorganisms against the logarithm of the ERY concentrations was plotted for each aeration period. Then, the concentration which inhibits the oxygen consumption was calculated to be by 50% (EC_{50}) (23).

Also, substrate utilization and the kinetic properties of the AS were evaluated by the plotting the respective OUR profiles for two series data and compared with control.

Kinetic parameter estimation using respirometry

The significant impact of antibiotics on the biodegradation of substrate is the reduction of oxygen consumption in the OUR experiments.

The respirometric methods used for estimation of kinetic constants include heterotroph yield constant (Y_H), half-saturation coefficient (K_S), and heterotrophic maximum growth rate ($\mu_{max,H}$) was described Grady et al (25) and do Céu Almeida and Butler (26). this method is based on measurement of OUR changes over the time of prolonged biomass aeration without any substrate addition in batch test reactors The OUR is related to biomass endogenous respiration (decay) only and the number of living (metabolizing) bacteria cells that is directly proportional to the decay rate of microorganisms (b_H). It is calculated through Eq. (10):

$$\text{Ln OUR Ln } ((0.8.b_H) \text{ X}_{T.t0}. f_{A,H}))-b_H.t \qquad (10)$$

The value of the decay constant (b_H) was determined using the linear regression technique as a slope of the curve obtained by plotting ln (OUR) versus aeration time. An f_D value of 0.2, applied in the calculations (fraction of biomass leading to debris), was taken from literature, then, the value of the active fraction of biomass ($f_{A,H}$) was calculated using Eq. (11):

$$f_{AH} = \frac{OUR_{t0}}{((0.8 \cdot b_H) \cdot XT_{t0} \cdot 1.12)} \qquad (11)$$

XT_{t0} was determined gravimetrically as a total suspended solids (TSS) concentration at the beginning of the experiment. Also, the conversion factor (1.12) for recalculation of the TSS mass concentration to the COD unit, was taken from literature.

The OUR area above the endogenous respiration level directly gives the amount of oxygen consumed in substrate depletion. Thus, with a known/predetermined amount of biodegradable substrate and DO changes (ΔO_2), the OUR curve was used to determine Y_H and/or inert COD components (25) by the following mass balance expression:

$$Y_H = 1 - \frac{O_{2O} - O_{2f}}{S_O - S_f} \qquad (12)$$

The kinetic parameters μ_H and K_s can be estimated, simultaneously, by the estimated values of active heterotrophs at biomass and biodegradable substrate concentrations in the batch reactor during respirometric experiments using Eq. (14):

$$\mu_H = SOUR \bullet \frac{Y_H}{1 - Y_H} \qquad (13)$$

where *SOUR* is the specific OUR (mgO$_2$/mg COD biomass.hr) at starting time.

$$SOUR = \frac{OUR}{X_H} \qquad (14)$$

By plotting the obtained μ_H values against the respective S_S values for the whole tests and using a nonlinear parameter estimation technique to fit the Monod kinetics equation, it is possible to determine the values of the half-saturation coefficient (K_S) and maximum growth rate ($\mu_{max,H}$).

Figure 1-3 present typical respirogram and related plots obtained during such ($\mu_{max,H}$, K_S, b_H and $f_{Active, H}$) determinations (27,28).

Results

DO changes were analyzed in biodegradation of peptone-meat extract, then, the acute impact of ERY was evaluated by the calculation of OUR reflected from the microbial system that was exposed to different ERY concentrations (0-1000 mg/L) for the first time as a non-biodegradable substrate.

The OUR profiles obtained from different experimental runs for total (series 1) and heterotrophic microorganisms (series 2) are given in Figures 4 and 5.

In order to determine the net impact of ERY and sludge on respiration, two-run without ERY and sludge were taken as a baseline (controls) in any setup.

According to experiment for both series (total and heterotrophic microorganisms), associated with biodegradation of peptone mixture in all runs (Table 1), the OUR level initially reached a peak value, then, followed by dropping to a stable endogenous respiration level with distinctly different rates corresponding to degradation of different COD fractions in the substrate.

The area under the OUR curve gives the total oxygen consumption, excluding the part related to endogenous respiration. According to OUR profiles for total microorganisms, ΔO_2 measured as 22.5, 12.7, 12.1, 11.4, and 11.2 mgO$_2$/L in batch reactors started with 0, 10, 100, 500, and 1000 mg/L ERY, respectively.

Also, for heterotrophic microorganisms, oxygen consumption was measured as 23.9, 11.25, 18.1, 9.1, and 7.6 mgO$_2$/L in batch reactors started with ERY dosing.
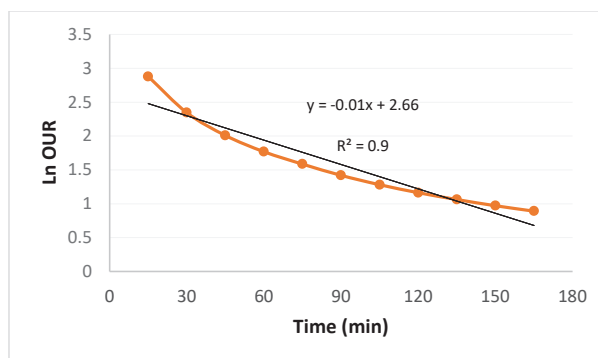

Figure 1. The plot of ln OUR versus time (t) during the determination of the b_H and f_{active}, the first run.
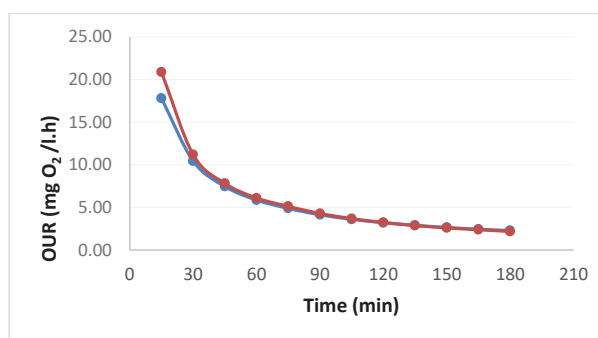

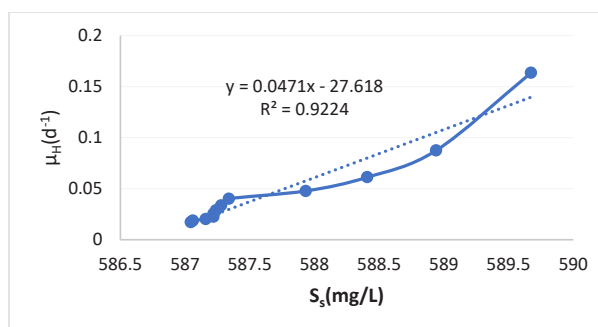**Figure 2.** Respirogram during estimation of K_S and μ_{max} (Run 3).

Figure 3. The plot of the relationship between the calculated values of heterotrophic growth rate and soluble substrate concentration. The line illustrates the Monod type curve fit to the experimental data. The estimated value of the half-saturation coefficient (K_S) is 9.8 mg COD/L, and the maximum growth rate (μ_{max}, H) is 5.5 d^{-1}.
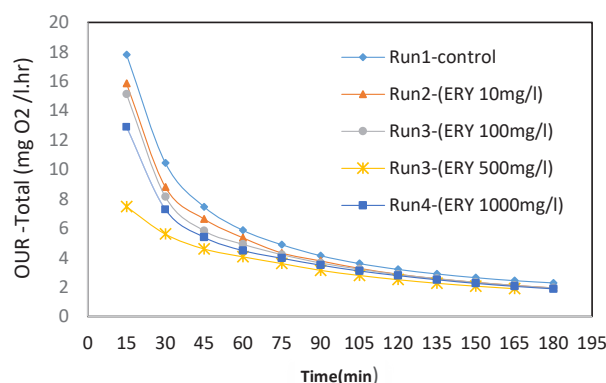

Figure 4. OUR profile for total microorganisms.

Table 1. Characteristics of batch tests

Runs	Antibiotic Concentration (mg.L^{-1})	Peptone COD (mg.L^{-1})	S_0/X_0 (mg COD.mg^{-1} VSS)	Antibiotic COD (mg.L^{-1})	Total COD (mg.L^{-1})
Run 1.1 (control)	0	50000	0.3	0	50000
Run 1.2	10	50000	0.3058	11.6	50011.6
Run 1.3	100	50000	0.358	116	50716
Run 1.4	500	50000	0.59	580	51180
Run 1.5	1000	50000	0.88	1160	51760

Evaluation of inhibitory impact for heterotrophic respiration and nitrification-linked oxygen uptake

The inhibition percentage of three different oxygen uptakes and effective concentration which inhibits the oxygen consumption by 50% (EC$_{50}$) were determined, namely total, heterotrophic, and that due to nitrification (Figures 5 and 6).

Kinetic parameter estimation

The OUR profile is a helpful instrument for interpreting the behavior and the metabolic functions of microbial systems in substrate utilization, biomass generation, and oxygen consumption processes.

The OUR profiles were evaluated for Kinetic parameter estimation. The results of kinetic parameter determination of heterotrophic bacteria in AS affected in ERY dosing (runs 1-5) were obtained using the methods described in the previous sections (Table 2). According to the results presented in Table 2, with increasing the concentration of ERY, b$_H$, f$_{A,H}$, and μ$_H$ decreased. However, the reaction kinetics for COD decomposition increased.

Discussion

Respirometry as the main experimental tool was used to evaluate biodegradation of peptone-meat extract mixture affected by different concentrations of ERY in the ISO 8192 inhibition test procedure (23). The respirometry results of batch tests for total and heterotroph microorganisms, showed that antibiotic addition has an inhibitory impact with a different pattern on biological processes, and alters the oxygen consumption rates and kinetic properties.

Thus, OUR profiles as reflections of biological reactions rate, are useful tools for evaluating the acute impact of different ERY concentrations on AS microorganisms. These profiles can be interpreted by comparing a control batch-test as a base-line, which does not contain ERY (16,29).

In another study by Amin et al, it was also found that the rate of oxygen uptake for the whole population of AS microorganisms decreases in the presence of antibiotics. The highest degree of inhibition for this amount of oxygen uptake was 28.8% (9).

In the present study, the results of respirometry batch tests for heterotrophic (Figure 5) and total microorganisms (Figure 4) showed that OUR level reaches a peak value initially in control reactors (20.88 and 17.8 mg O$_2$/L.h) due to the utilization of readily biodegradable COD fraction in peptone mixture. Then, dropped with different slops, until OUR profiles reached the initial endogenous respiration (1.76 and 2.28 mg O$_2$/L.h) by continuous hydrolysis of other COD fractions with different degradation properties. The highest degree of inhibition for this OUR was 28.8%.

In addition, a slight decrease in the rate of inhibition of oxygen uptake was observed for heterotrophic bacteria (13.7). Nitrifying bacteria were the most sensitive to ERY.

The degree of inhibition of oxygen uptake in the presence of this antibiotic for the first phase nitrifying bacteria reached 40%-64.5%, while for the second phase bacteria, this value was 28.2%-62%.

Also, the other curves started with ERY concentration of 1-1000 mg/L, the OUR level initially has a lower peak, then,

Table 2. Results of determination of the decay coefficient (b$_H$) and an active fraction (f$_{A,H}$) of heterotrophic biomass in biofilm, yield coefficient of X$_H$ (Y$_H$) and maximum growth rate for X$_H$ (μ$_H$)

Runs	b$_H$(d^{-1})	f$_{A,H}$	Y$_H$ (mg VSS/mg COD)	μ$_H$ (d^{-1})
Run 1- concentration = 0	2.66 (0.91)	0.44	0.4945	0.047 (0.92)
Run 2- concentration = 10	2.49 (0.82)	0.37	0.4953	0.027 (0.88)
Run 3- concentration = 100	2.39 (0.91)	0.34	0.4959	0.016 (0.97)
Run 4- concentration = 500	2.31 (.091)	0.31	0.4975	0.008 (0.81)
Run 5- concentration = 1000	2.27 (0.91)	0.3	0.4983	0.0049 (0.82)

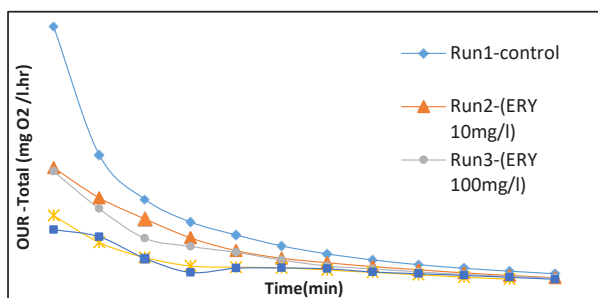*Note*: Correlation coefficients (R^2) are given in brackets.

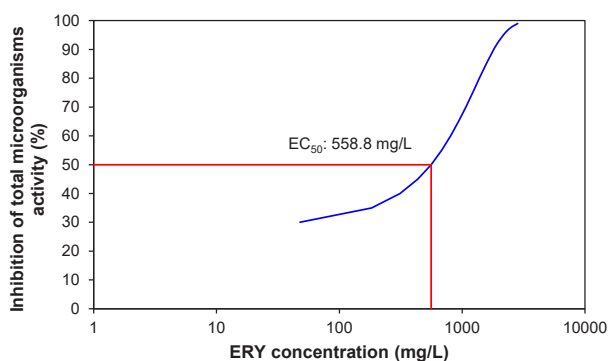**Figure 5.** OUR profile for heterotrophic microorganisms.

Figure 6. Dose-response curve for total microorganisms.

continues to drop in all batch tests (total and heterotroph) with distinctly decreasing rates with increasing ERY dose.

The peak OUR value in the control test for heterotrophic microorganisms was higher than that for the total microorganism, but in other batch tests, the contained ERY maximum OUR levels were lower.

Especially, the difference between the two series OUR profiles is related to the net impact of nitrifies, that are present in total group bacteria.

In spite of the experimental results of previous studies on the sensitivity of nitrifies to toxic materials, these bacteria were less sensitive to ERY in comparison to heterotrophic bacteria.

Similarly, the studies by Pala-Ozkok et al on ERY and tetracycline (TET), showed that antibiotic addition affected the shape of the OUR profiles in both series tests (ISO 8192 and respirometry) for ERY, by reducing the DO consumption (24,25).

The area under the OUR curve as the total oxygen consumption, for total and heterotrophic microorganism, dropped with increasing ERY dose from 0 to 1000 mg/L, indicating that antibiotic addition reduced oxygen consumption and affected curves shape for all groups of microorganism.

The comparative evaluation of the calculated results for both series of batch tests showed that minimum oxygen consumption occurred in 1000 mg/L for total (11.2 mg O_2/L) and heterotrophic (7.6 mg O_2/L) microorganisms. The interesting feature of the experimental results is that these levels are lower than the other ERY concentrations

in the last group (without any nitrifiers).

Evaluation of the obtained OUR profiles showed that while all available foreign substrates were removed from the solution, the addition of antibiotics significantly reduced the amount of oxygen consumed. These results showed that a different part of the peptone mixture was blocked by the antibiotic and did not participate in the ongoing microbial growth mechanism.

Therefore, it seems that the reduction of total oxygen consumption associated with microbial metabolism is the main sign of the inhibitory effect of the selected antibiotics (7).

The OUR reduction was observed in the range of 85%–87% and 68-90% for total and heterotrophic microorganisms, respectively, with ascending rate of 0 to 1000 mg/L ERY.

Also, in the study of Ozkok et al on two concentrations of sulfamethoxazole, TET, and ERY antibiotics (50 and 200 mg/L) by respirometry and ISO test methods, the results of both methods showed that the addition of antibiotics affected OUR profiles (12%-37% and 15%-59% reduction for 50 mg/L ERY, respectively) but, the oxygen consumption increased by increasing dose of antibiotics in respirometry. In the ISO test method, because of the nature of the test, this effect of antibiotics was lower observed by increasing the initial dose of antibiotics, in the present study ERY (7).

However, addition of ERY totally accelerated endogenous respiration that could be explained by higher maintenance energy required for generating specific inactivating enzymes to prevent the toxic effects of ERY (24,30).

The discrepancies between oxygen consumption, OUR profile, and inhibition percentage in these researches can be due to the difference between sludge origin and its history.

In this context, the study of Pala-Ozkok et al on the acute effect of ERY on two AS aged 2 and 10 days, showed that in control batch reactors started with the biomass acclimated to different sludge ages generated totally different OUR curves, suggesting similarly different process kinetics for utilization of the same substrate. Also, addition of ERY (50 mgL⁻¹) to the batch reactors completely changed the shape of the OUR profiles, which significantly reduced the DO utilization for the two series (24).

Louvet et al examined the effects of 0-10 mg/L ERY on two different origins AS from WWTP in French. They found that ERY inhibited the specific evolution rate of COD (79%, SD 34%) and the specific evolution rate of N–NH₄ (41%, SD 25%), but its effect on nitrification was variable because of the sludge origin (15). Also, nitrifiers were sensitive to ERY at a concentration higher than 1 mg/L. This observation can be related to the fact that, most nitrifying bacteria are known as gram-negative, and ERY is more active against gram-positive bacteria.

In all batch reactors started with the biomass acclimated to sludge age of 10 days, addition of ERY generated a totally different OUR curve, suggesting similarly different process kinetics for utilization of the same substrate (Table 2). As shown in Figures 4 and 5, the OUR descent after the initial peak gives a clearer picture of different COD fractions involved (24,25).

According to the adopted model that defines OUR in terms of the well-known Monod-type growth rate equation, regardless of endogenous respiration in the diagram, the mass balance was adapted and the total COD was considered biodegradable. Thus, the OUR curves were used to determine Y_H, $\mu_{max, H}$, b_H, and $f_{A, H}$ (31,32).

For this purpose, the Monod kinetic constant for heterotrophic bacteria by means of OUR profile, indicated variable process kinetics for the control reactor and the others (Runs 1-5) containing ERY (Table 2).

In this context, the applicable Y_H value with no intracellular storage assumption (28), was calculated to be about 0.49 mg cell COD mg^{-1} COD (mg VSS/mg COD) in control and the other batch tests containing different ERY dosages. The effect of ERY on nitrification varied depending on the origin of the AS and differences in its composition (9,10).

This value is slightly lower than the range of 0.60–0.64 mg cell COD/mg COD commonly associated with different sludge properties (31). ERY concentration of 10 mg/L inhibits the COD of 79% (standard deviation 34%) and the specific N-NH$_4$ evolution rate of 41% (standard deviation of 25%).

Also, ERY dosing has an apparent negative impact on microbial growth with the reduced maximum specific heterotrophic growth rate (μ_H).

A significant effect of ERY dosing was observed on the endogenous respiration rate (b_H), which gradually decreased from 2.66 d^{-1} in the control test to 2.27 d^{-1} in the reactor containing the maximum ERY concentration. Similar to b_H, the active fraction ($f_{A,H}$) apparently decreased from 0.44 to 0.3 by increasing ERY dose, because of the inhibition effect.

Thus, ERY addition reduced the DO consumption and OUR rate in accordance with biodegradable COD utilization, and finally, as an inhibitor, would affect the process kinetics, either by lowering the maximum growth rate (μ_{Hmax}), or by reducing the activity of biomass, that is reducing X_H. These observed changes in the OUR profiles could be the result of changes in biomass caused by the bactericidal effect of antibiotics (7).

In the study of Pala-Ozkok et al on the effect of ERY dosing on biomass with different sludge ages, the endogenous respiration rate (b_H), increased from 0.20 d^{-1} (2 days) and 0.10 d^{-1} (10 days) in the control reactor to 0.40 d^{-1} and, to 0.20 d^{-1} and 0.24 d^{-1} for 50 mg ERY L^{-1} and 200 mg ERY L^{-1} addition, respectively (24).

The high values for b_H are noticeable signals for starting

a mechanism overcome to the toxic effects of ERY by the microbial community (24). In the present study, these values can be originated from the nature of this respirometry and the length of batch tests.

Similar to previous studies (7,33), in accordance with culture history changes, the microbial consortium altered that fed with the same substrate.

The macrolides, especially ERY as a protein synthesis inhibitor, have been found to significantly inhibit the main biological process for removal of nutrients. For example, increasing concentrations of the ERY negatively affected nitrification and denitrification and microbial communities, particularly filamentous bacteria as floc backbone. Thus, the microbial community dynamics is suggested as the main reason for observing variable process kinetics (34).

In the present study, according to the percentage inhibition results (Figures 6-8), the highest calculated half-maximal effective concentration (EC$_{50}$), was related to nitrifying (1243.1) microorganisms, followed by total (558.8) and heterotrophic (269.4) microorganisms, respectively. Also, a concentration of 1000 m/L showed the highest inhibition (100%).

Similarly, the results of the study of Tomska and Worwąg on the effect of ERY on biochemical activity of AS microorganisms, showed that OUR declined for total microorganisms of AS in the presence of the antibiotic. Also, the lowest value of dehydrogenase activity was
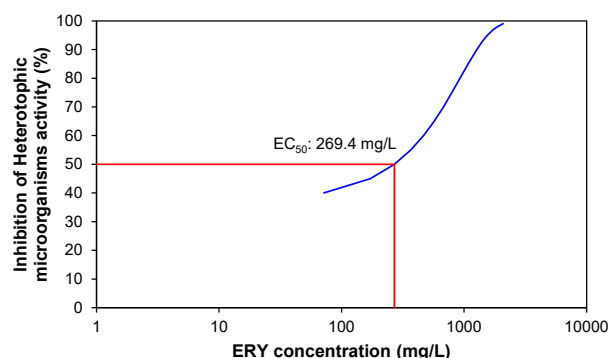

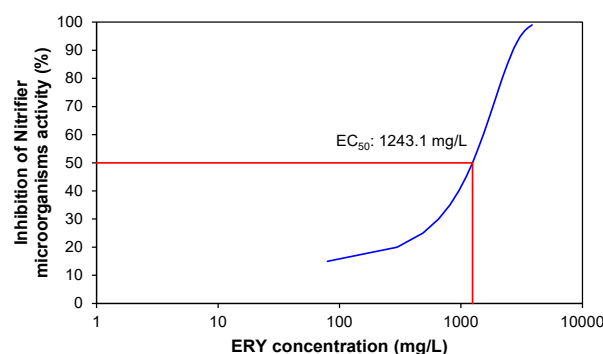**Figure 7.** Dose-response curve for heterotrophic microorganisms.

Figure 8. Dose-response curve for nitrifying microorganisms.

related to ERY concentration of 150 mg/L with a 33.7% inhibition degree. But, an insignificant decline in the degree of inhibition of OUR was observed for heterotrophic bacteria (13.7%). The most sensitive to ERY were nitrifying bacteria. The degree of inhibition of OUR in the presence of this antibiotic for nitrifying bacteria of the first ammonia-oxidizing bacteria and the second phase nitrite-oxidizing bacteria reached 40%–64.5% and 28.2%–62%, respectively (35).

ERY is more active against gram-positive bacteria, but most nitrifying bacteria are known as gram-negative, thus, nitrifying group was less inhibited than heterotroph and total bacteria in spite of more sensitivity to toxic materials (15).

The study of Katipoglu-Yazan et al on the effects of ERY and TET on biological processes kinetics indicated that both steps of nitrification were affected by ERY, but TET inhibited and retarded nitrification kinetics at 50 mg/L and stopped nitrite oxidation at 200 mg/L. Also, ERY and TET partially inactivated the heterotrophic community in the culture, increased substrate storage, and accelerated endogenous respiration to overcome antibiotics toxic effects (24,36).

In this context, Halling-Sørensen investigated the effects of Tylosin on AS, as a macrolide antibiotic which is similar to ERY, and EC_{50} of 54.7 mgL^{-1} was determined for this antibiotic (37).

In the study of Aali et al on ERY toxicity at a concentration of 1–300 mg L^{-1}, ERY has been found to significantly inhibit ammonification, nitrification, and denitrification at concentrations higher than 20 mgL^{-1}. Also, floc disintegration was observed in the presence of ERY because of its susceptibility to surfactant properties and cell lysis (8).

The difference between EC_{50} in different researches can be related to respirometry test technique, culture history, and microbial composition, and probably antibiotic-resistant bacteria to antibiotic (38).

Conclusion

In this study, the inhibition of AS microorganisms by ERY was confirmed in batch tests at high concentrations (0-1000 mg/L) in the range of what has been found in pharmaceutical and hospital WWTPs.

The inhibitory impact of ERY was observed as a decrease in the amount of oxygen consumption by OUR profiles in the rapid respirometric method (ISO 8192) and offered a novel insight for the acute inhibitory impact of this antibiotic.

The half-maximal effective concentration (EC_{50}), for total, heterotrophic, and nitrifying microorganisms was observed at 558.8, 269.4, and 1243.1 mg/L of ERY, respectively. But in spite of continuous OUR profiles, the nature of the ISO test does not provide additional clues for explaining the results obtained for the inhibitory action of antibiotics on heterotrophic and autotrophic microorganisms.

The culture history and its origin affected the nature of inhibition, indicating different process kinetics for a faster-growing microbial community.

The study underlined the importance of assessing the substrate fraction changes as COD and of microbial culture history, as an integral part of investigations on the adverse effects of chemicals (e.g. ERY in the present study) on the process kinetics defining substrate utilization in biological systems. Thus, additional studies are now underway to investigate the inhibitory effects of ERY on a variety of biomass and real effluent properties under continuous operation tests and pilot-scale WWTPs.

Ethical issues
The study protocols were approved by the Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran (Ethical code: IR.MUI.RESEARCH.REC.1397.300).

Competing interests
The authors declare that they have no conflict of interests.

Authors' contributions
All authors contributed and were involved in the problem suggestion, experiments design, data collection, and manuscript approval.

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