Effects of combined antibiotics on the biomass stability and the occurrence of antibiotic-resistant bacteria in activated sludge used for domestic wastewater treatment

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Abstract

The main objective of this study was to evaluate the effects of combined antibiotics on biomass stability and the occurrence of antibiotic-resistant bacteria (ARB) in activated sludge (AS) used for domestic wastewater treatment. Two aerobic lab-scale reactors were operated: one for the experimental approach (R1) and the other as a control (R2). In R1, two exposures were conducted with combined antibiotics of amoxicillin (AMX), ciprofloxacin (CIP) and ceftriaxone (CTX) at concentrations of 32, 4, and 2 µg/L, respectively, during exposure 1 (E1), and 64, 8, and 4 µg/L, respectively, during exposure 2 (E2). In these cases, removal efficiencies, oxygen uptake rate (OUR), specific oxygen uptake rate (SOUR) and ARB occurrences were evaluated. Within the results of this study, a significant effect on the AS biomass in terms of performance and kinetic parameters was observed with values below 10%, 20 $mgO_2/L \cdot d$ and 100 $mgO_2/gVSS \cdot d$ for organic matter removals, OUR and SOUR, respectively, during E1. However, during E2, AS biomass performances and kinetic parameters improved, with a 50% increase in these values. Moreover, ARB occurrences were only detected during E2, with abundances between 6.6 – 8.2 $x10^2$ CFU/mL. These findings suggest that the re-expose of AS biomass to combined antibiotic concentrations induced bacterial adaptation to this selective pressure resulting in the recovery of the heterotrophic activity and improved removal efficiency.

Keywords: antibiotics, antibiotic-resistant bacteria, activated sludge biomass, respirometry, kinetic parameters.

1. Introduction

Wastewater treatment plants (WWTPs) play a crucial role in controlling pollution in aquatic ecosystems by removing organic matter and nutrients, thus preventing eutrophication [1]. However, in the context of antibiotic resistance (AR) dissemination, WWTPs act as reservoirs because they are not designed to remove antibiotics present in wastewater Furthermore, during biological treatment, the microbial growth conditions are optimal for facilitating the various mechanisms that promote the spread of AR [2–5].

The presence of AR elements (antibiotics, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs)) in wastewater also poses a problem for the microbial activity in biological treatment processes [6]. In the case of activated sludge (AS), one of the most widely implemented biological technologies in WWTPs worldwide, the activity of heterotrophic bacteria, which aerobically degrade organic matter, is affected by antibiotic concentrations [7]. Kumar et al. [8] investigated the impact of different amoxicillin (AMX) concentrations on the activity of aerobic biomass. This study revealed that chemical oxygen demand (COD) removals decreased when reactors were exposed to AMX concentrations between 500 to 2000 mg/L. However, the biomass activity was recovered and the removal efficiencies of COD increased to 93% after 35 days of operation without AMX concentration. Same behavior was observed in He et al. [9] where a lab-scale sequencing batch reactor was operated under aerobic condition, and it was exposed to different chlortetracycline (CTC) concentrations (0.05 – 1 mg/L). The results showed that the performances of COD, total nitrogen (TN) and total phosphorus (TP) were affected by the presence of the antibiotic achieving removal efficiencies between 40 – 60%. Other studies from He et al. [10] and Li et al. [11] revealed that anaerobic biomass was also inhibited by oxytetracycline (OTC) decreasing the removal efficiency of COD from 90% to 70%.

The study of microbial activity in WWTPs is often approached through the analysis of microbial communities using high throughput sequencing tools [12,13]. While this method is valuable and insight, it requires highly qualified personnel with expertise in bioinformatics and data processing. To assess the effect of emerging contaminants such as antibiotics on biomass activity in a more straightforward manner, respirometry is a useful technique for achieving this purpose [14]. Pala-Ozkok et al. [15] studied the kinetic impacts of exposure to

sulfamethoxazole (SMX) on an AS biomass. The results of oxygen uptake rate (OUR) showed that this value decreased from 160 mg/L·h to 106 mg/L·h when SMX concentration of 50 mg/L was added to the reactor. In the same line, Pala-Ozkok et al. [16], who evaluated the impacts of tetracycline (TET) on an AS biomass, found that OUR dropped from 160 mg/L·h to 92 mg/L·h when the biomass was fed with a TET concentration of 50 mg/L for 120 days.

The occurrence of antibiotics in wastewater may also exert a selective pressure to the biomass generating the growth of ARB and the spread of ARGs into the environment [17]. Neyestani et al. [18] studied the occurrence and proliferation of ARB during biological wastewater treatment with different influent antibiotic concentrations. They observed that higher influent antibiotic concentrations (400 - 3200 mg/L) may increase the prevalence of ARB during the biological treatment. On the contrary, Slipko et al. [19] observed no significant effect of increasing ciprofloxacin (CIP) concentrations on ARB in a AS reactor. They attribute their results to the possibility that the biomass was adapted to wastewater conditions and was not affect by tested concentrations (0.0001 – 0.1 mg/L).

Although studies have shown that the presence of antibiotics affects the kinetics of AS biomass and the abundances of ARB, reactor operations are often conducted under controlled conditions using synthetic influent to feed the reactors, with antibiotic concentrations higher than those typically found in domestic wastewater (~0.0001 mg/L) [2]. Moreover, the effect of combined antibiotics on the activity of AS biomass has been little explored. In this context, the main objective of this study was to evaluate the effects of combined antibiotics on biomass stability and the occurrence of antibiotic-resistant bacteria (ARB) in activated sludge used for domestic wastewater treatment. The AS biomass was evaluated under two exposures to combined antibiotics: AMX, CIP, and ceftriaxone (CTX) at concentrations of 32, 4, and 2 µg/L, respectively, during exposure 1 (E1), and 64, 8, and 4 µg/L, respectively, during exposure 2 (E2). The effects of combined antibiotics on the removal efficiencies of COD, BOD_5, TN, and ammonium nitrogen (NH_4^+-N) were determined, and kinetic parameters such as oxygen uptake rate (OUR) and specific oxygen uptake rate (SOUR) were also considered. Furthermore, ARB abundances were measured before and after E1 and E2 to evaluate the effects of antibiotic resistance dissemination during the AS process.

2. Materials and Methods

2.1. Reactors setup and operation

Two lab-scale reactors were set up for this study: one for the experimental approach (R1) and the other as a control (R2). The sequential treatment system consisted of an aeration tank followed by a settler tank, with volumes of 1 L and 0.7 L, respectively. The influent for both systems was raw wastewater from the Hualqui WWTP in the Biobío Region, Chile (36°59'26.93" S and 72°56'47.23" W), and was fed into both reactors using a pump. Each reactor was inoculated with AS biomass from the same WWTP and operated for 245 days at an average temperature of 20 ± 4.8°C, with a hydraulic retention time (HRT) of 8 hours. Dissolved oxygen (DO) was maintained at 2.0 mg/L. The organic loading rate (OLR) and flow rate were 0.6 kg $BOD_5/m^3 \cdot d$ and 2.5 L/d, respectively, for both reactors. Wastewater was recirculated from the settler to the aeration tank at a flow rate 1.5 times higher than the inflow.

The experimental approach was divided into three stages with two punctual exposures to antibiotics. The first stage involved the stabilization of both reactors, where the systems were acclimatized and operated under normal conditions for 202 days. This stage was longer than expected, as both reactors experienced *bulking* problems and had to be re-stabilized. Then, concentrations of 32 µg/L AMX, 4 µg/L CIP, and 2 µg/L CTX were added to the influent of R1. After exposure 1 (E1), the reactor was operated under normal conditions for 37 days without the addition of antibiotics. The biomass was then re-exposed to the combined antibiotics, with concentrations doubled during the second exposure (E2) (64 µg/L AMX, 8 µg/L CIP, and 4 µg/L CTX). After E2, R1 was operated under normal conditions for further 11 days. During all stages, influent (I) and final effluent (FE) water samples as well as biomass samples, were collected twice a month. Figure 1 summarizes the experimental approach used in this study.

Figure 1

2.2. Determination of physicochemical parameters

In situ parameters such as pH, temperature (T), electrical conductivity (EC), DO and oxidation-reduction potential (ORP) were measured in water and biomass samples once a

week using OAKTON portable equipment (PC650-480485, OAKTON, USA), except for DO that a portable oxygen sensor (HANNA OXI 330i/set HI 9146-04, HANNA Instruments Inc., USA) was used. Additionally, turbidity was also determined in water samples using a portable waterproof turbidimeter (OAKTON T-100, OAKTON, USA).

For water samples (I and FE) of both reactors, COD, biological oxygen demand (BOD_5), TN, NH_4^+-N, nitrogen as nitrate (NO_3^--N), nitrogen as nitrite (NO_2^--N), TP and phosphorus as phosphate (PO_4^{-3}-P) were determined based on the protocols of Standard Methods [20]. In the case of biomass samples, total and volatile suspended solids (TSS and VSS) were also measured, and the sludge volumetric index (SVI) was also calculated as described by Neumann et al. [21].

2.3. Respirometric analysis

The heterotrophic activity of the AS biomass from both reactors was evaluated through respirometric analysis, where the OUR and SOUR were determined using an exogenous biodegradable substrate (sodium acetate). A biological oxygen monitor (BOM), YSI 5300 System (YSI Incorporated Life Sciences, Yellow Spring, OH, USA), was used with an air-tight respiration vessel fitted with a YSI 5231 DO probe. The vessel was continuously stirred and thermally controlled at 25°C, using a biomass concentration of 2.0 g VSS/L, previously washed with phosphate buffer. OUR was determined by linear regression from the slope obtained by plotting DO concentration against time, and SOUR was calculated using the VSS value measured in the assay [22,23]. During the stabilization stage, this analysis was carried out biweekly in both reactors. For E1 and E2 in R1, the respirometric analyses were performed the day before, the same day, and the day after the exposures.

2.4. Quantification of Antibiotic-Resistant Bacteria

The abundances of antibiotic-resistant bacteria (ARB) were determined in water samples (I and FE) from R1 during stabilization, E1, and E2. This analysis was not performed in R2. This analysis followed the protocols described by Leiva et al. [24], where the plate count technique was based on the ability of bacteria to grow in the presence of AMX, CIP, and CTX. MacConkey agar was used as the culture medium, and plates were inoculated with water samples and incubated at 30°C for 24 hours. The plates were supplemented with the

most common antibiotics used in Chile and previously reported in WWTPs, such as AMX, CIP, and CTX (OXOID, Thermo Scientific), at concentrations of 2 mg/L, 32 mg/L, and 4 mg/L, respectively [24,25] which represent the breakpoint values for defining resistance to these antimicrobial agents [26]. Control plates without antibiotics were also inoculated, and only plates with 30 to 300 colonies were considered for enumeration.

2.5. Statistical Analysis

The effects of E1 and E2 on the AS biomass in terms of removal efficiencies, OUR, SOUR, and ARB were analyzed using RStudio version 4.4.1, with a significance level of $p = 0.05$. The data were tested for normality and homogeneity of variance using the Shapiro-Wilk test and the Fligner-Killeen test, respectively. ANOVA was performed on data with a normal distribution, while the Kruskal-Wallis test was applied to data without a normal distribution. Finally, principal component analysis (PCA) was conducted using the principal function from the "psych" R package.

3. Results and Discussion

3.1. Effects of combined antibiotics on the removal efficiencies of organic matter and nitrogen forms

Table 1 presents the physicochemical characterization of the influent during different stages of operational time. The concentrations correspond to the average value during these stages. Regarding the parameters, these values were within the ranges reported in the literature for domestic wastewater with COD, BOD_5, NH_4^+-N, TN, TP and PO_4^{-3}-P concentrations that varied between 740 – 45 mg/L, 500 – 14 mg/L, 80 – 10 mg/L, 80 – 20 mg/L, 20 – 4 mg/L and 15 – 4 mg/L, respectively [1,24,27,28]. No significant differences in the influent during different stages (stabilization, E1 and E2) were observed ($p > 0.05$).

Table 1

In terms of the effects of combined antibiotics on R1 performance, Figure 2 shows the removal efficiencies of organic matter (Fig.2a) and nutrient forms (Fig.2b). During the stabilization stage, the removal efficiencies of COD, BOD_5, NH_4^+-N, and TN varied between 41 – 99%, 65 – 90%, (-14) – 67%, (-9) – 26%, respectively. These values decreased significantly during E1 for COD and BOD_5 reaching performances of 42% and (-37%),

184 respectively ($p < 0.05$). For BOD_5 removals, the effect of antibiotic exposure was higher than
185 for COD removal. This behavior may be related to the availability of oxygen at this stage
186 which affects the biodegradation process [15]. After 37 operational days, the organic matter
187 degradation in R1 recovered achieving removal efficiencies above 90%. These results
188 indicate that organic matter removals were initially affected by combined antibiotics (AMX,
189 CIP and CTX) exposure, but the biomass had the capacity to recover its degradation activity.
190 This is demonstrated by the fact that, following E1, the system once again achieved removal
191 efficiency similar to the stabilization period. The same behavior was observed by Kumar et
192 al. [8] who investigated the recovery potential of aerobic biomass after exposure to AMX and
193 found that the removal efficiencies of COD decreased to 23% during exposure but recovered
194 to 93% during the recovery phase (35 days), similar to pre-exposure levels.

195 ***Figure 2***

196 Moreover, an interesting effect was observed when R1 was re-exposed to the double
197 antibiotic concentrations (E2). For COD and BOD_5, the removal efficiencies remained in the
198 80 – 90% range, showing that the organic matter degradation was not affected by combined
199 antibiotics during E2. In this case, the biomass likely developed resistance mechanisms under
200 selective pressure during E1, allowing it to counteract the effects of antibiotics during re-
201 exposure. Several studies support the idea that bacteria and antibiotics coexist in the biomass
202 of WWTPs, and this complex environment can promote the transfer of resistance
203 mechanisms and the adaptation of the biomass [29–32].

204 On the other hand, the removal efficiencies of NH_4^+-N and TN during the exposures (E1 and
205 E2) remained within similar ranges to those achieved during the stabilization phase, with
206 values fluctuating between 50 – 75% and 12 – 35%, respectively (Fig2.b). In this case, no
207 significant effects of combined antibiotics on nitrogen removal were observed ($p > 0.05$).
208 Figure 1S shows the removal efficiencies of these parameters in R2. Several studies have
209 indicated that the nitrification process, one of the main mechanisms for NH_4^+-N in AS, can
210 be inhibited by the antibiotic exposure [9,12,16,17]. However, many of these reports used
211 antibiotics concentrations in the order of mg/L. In this study, the concentrations did not
212 exceed 100 µg/L, which are relatively low. Furthermore, De Sotto et al. [33] studied the
213 effects of TET, ampicillin (AMP) and SMX on the nutrient removals in AS samples taken

from two distinct membrane bioreactor (MBR) systems (reciprocation MBR vs. air-scouring MBR). In their case, the AS from the continuous air scouring flow MBR showed removal efficiencies close to 100% under the acute antibiotic exposure.

To further confirm the effect of antibiotic exposures (E1 and E2) on removal efficiency, a PCA was performed. Figure 3 shows the results of this analysis where the principal components 1 and 2 (PC1 and PC2) explained 37.6% and 19.6% of the total variance, respectively. These results indicate that these two components account for 57.2% of the total variance and it is possible to analyze the association between water samples, stages, and parameters. Regarding the association between variables, PC1 reveals a strong association between COD and BOD_5 concentrations with values of -0.46 and -0.44, respectively (see Table 1S). In PC2, this component is explained by the association between NO_3^--N, NO_2^--N, TP and PO_4^{-3}-P concentrations. These results are expected because organic matter and nutrients are correlated in wastewater treatment. Nitrates participate in the nitrification process in activated sludge and 90% of the TP content corresponds to PO_4^{-3}-P [15]. The score plot showed that the water samples were clustered into three groups according to the different stages: stabilization, E1, and E2. Water samples from the stabilization stage are spatially separated from those of E1 and E2. Furthermore, E1 and E2 remained separated from each other. These results are consistent with the trends observed in organic matter removals and reveal that combined antibiotics had a significant effect on R1 performance ($p < 0.05$). Likewise, this analysis also demonstrated that E1 and E2 had differing effect on the reactor's degradation activity.

Figure 3

3.2. Effects of combined antibiotics on the kinetic parameters of the AS biomass

Table 2 shows the biomass characterization of R1 and R2. In terms of OUR and SOUR averages during the operational time, these parameters were 14% and 47% higher in R1 than R2, with values of 144.3 $mgO_2/L \cdot d$ and 303.2 $mgO_2/gVSS \cdot d$, respectively. These results indicate that oxygen uptake and consequently biomass had a higher activity in the reactor exposed to combined antibiotic concentrations compared to control reactor (R2). Regarding SVI, R1 and R2 showed values of 134.6 mL/gTSS and 184.0 mL/gTSS, respectively suggesting a sludge with good settling characteristics [34].

Table 2

Figure 4 shows the heterotrophic activity of R1 biomass (Fig.4a) and the relation between SVI and food/microorganism ratio (F/M) in R1 (Fig.4b). Regarding the behavior of OUR and SOUR during the operational time, these parameters fluctuated between 38.2 – 277.3 $mgO_2/L \cdot d$ and 26.9 – 391.0 $mgO_2/gVSS \cdot d$, respectively, in the stabilization stage (Fig.4a). When the biomass was exposed to combined antibiotic concentrations (E1), these parameters exhibited variable trends, reaching values above 100 $mgO_2/L \cdot d$ and 600 $mgO_2/gVSS \cdot d$ and values below 20 $mgO_2/L \cdot d$ and 100 $mgO_2/gVSS \cdot d$ for OUR and SOUR, respectively. In E2, similar variations were observed, where OUR and SOUR decreased to 89.6 $mgO_2/L \cdot d$ and 107.1 $mgO_2/gVSS \cdot d$, respectively, after the antibiotic exposure, then increased to values above 800 $mgO_2/L \cdot d$ or $mgO_2/gVSS \cdot d$. These results suggest instability in the heterotrophic activity in the biomass during the exposures.

Figure 4

In the same line, the relation between SVI and F/M ratio in R1 was studied in Fig.4b. It is important to mention that the upper left quadrant of the plot represents a sludge with good quality and sedimentation characteristics (SVI > 50 mL/gTSS and 0.3 < F/M < 0.9 $kgDBO_5/kgVSS \cdot d$, respectively). In the case of data from the stabilization stage, the relation was located in this quadrant (black squares). However, a different trend was observed for data from E1 and E2. In both cases, the F/M ratio achieved values of 18.3 $kgDBO_5/kgVSS \cdot d$ and 1.7 $kgDBO_5/kgVSS \cdot d$, respectively, and sedimentation problem such as filamentous *bulking* was observed during E1 and E2 (data not shown). Table 3 shows the kinetic parameters of R1 during heterotrophic activity. The same tendency of biomass instability during antibiotic exposures was observed. In R1, the lower values of OUR_{end} and OUR_{exo} were achieved with a decrease of 49% compared to those reported during the stabilization stage. For OUR_{end} and OUR_{exo} in R2, they increased compared to R1 achieving similar results that the stabilization stage with values of 10.8 $mgO_2/L \cdot h$ and 24.3 $mgO_2/L \cdot h$, respectively.

Table 3

This study demonstrated that the activity of AS biomass was affected by the presence of combined antibiotics (AMX, CIP and CTX). These findings align with previous studies by

Pala-Ozkok et al. [15,16], who investigated the effects of TET and SMX on the kinetic parameters of AS biomass. In their work, OUR profiles decreased by 42% and 33% following exposures to TET and SMX, respectively. Similarly, Faria et al. [35] reported that AMX exposure negatively impacted growth rate, reducing it by 74%. In terms of evaluating the effects of exposures, the results of kinetic parameters and removal efficiencies demonstrated that E1 had a higher negative impact on biomass activity than E2. In this case, the endogenous activity decreased, and the specific oxygen consumption increased (SOUR: 57.1 $mgO_2/gVSS \cdot d$) showing a microbial overactivity in response to a toxic environment and limited food availability[33]. Conversely, during E2, the endogenous activity increased. Pala-Ozkok et al. [16] observed a similar trend, attributing it to maintenance energy demand driven by presence and production of ARGs.

3.3. The effects of combined antibiotics on the antibiotic-resistant bacteria (ARB) abundances

Figure 5 shows the abundances of ARB in water samples (I and FE) during stabilization stage, E1 and E2. In general, the ARB abundances tended to increase from the stabilization stage to E2 showing an effect of the antibiotic addition. In the case of I samples, the addition of combined antibiotics to the wastewater impacted ARB abundances, with ARB resistant to AMX and CIP increasing by 67% and 38%, respectively. This result indicates that the I samples had a baseline abundance of these ARBs, which is expected due to their origin from domestic wastewater, previously reported to contain AR elements [24]. In this study, the abundances of ARB resistant to AMX, CTX, and CIP was determined in 20 rural WWTPs with values ranging from 10^5 – 10^6 CFU/mL. The WWTP from which the influent and biomass in the present study were obtained, was located in the same area as these 20 rural WWTPs, suggesting that these samples could contain AR elements. Moreover, Reyes-Contreras et al. [36] analyzed the occurrences of emerging contaminants in the same WWTP and determined concentrations of some antimicrobial agents such as triclosan suggesting that AR occurs in this system. The ARB abundances in I samples varied between $9.9x10^2$ – $4.3x10^3$ CFU/mL during the operational period (from stabilization to E2). For FE samples, the abundances fluctuated between 6.6 – $8.2x10^2$ CFU/mL during E2, with reductions between 0.3 – 0.6 log unit. At the stabilization and E2 stages, some abundances of ARB

303 resistant to AMX, CIP, and CTX were below the detection limit (<3.3 CFU/mL). These
304 values are within the ranges reported in the literature, which varied between 10^3 – 10^6 and
305 10^2 – 10^3 in I and FE samples from WWTPs, respectively [2,24,37–39]. Regarding ARB
306 resistant to CTX in I samples, only E1 and E2 showed positive values, indicating that
307 resistance was acquired due to the selective pressure exerted by the antibiotics. For FE
308 samples, ARB abundances were only observed during E2, except for ARB resistant to CIP
309 that had occurred during E1. These results showed that the combined antibiotics influenced
310 the occurrences of ARB in R1 and therefore, the spreading of AR elements due to the
311 selective pressure.

312 During the operational time, AR dissemination occurs in R1. With the addition of combined
313 antibiotics, the ARB abundance increased from the stabilization to E2, showing that the
314 reactor develops and spreads AR to adapt to the antibiotic concentrations. These findings
315 align with those reported for the removal efficiency and the biomass activity. During E1, the
316 AS biomass was destabilised showing variable OUR and SOUR, as well as a decrease in
317 organic matter removals. Conversely, the biomass began to recover its heterotrophic activity
318 and performances in E2. The occurrence of ARB during this exposure can be linked to this
319 behavior. The biomass developed and disseminated AR in the microbial environment to face
320 and survive a new exposure to antibiotics. Similarly, Neyestani et al. [18] demonstrated that
321 higher influent antibiotic concentrations led to a greater prevalence of AR and ARB isolates
322 were generally resistant to antibiotic concentrations 32 higher than their baseline. This
323 demonstrates that biomass can acquire AR mechanisms and adapt to antibiotic concentrations
324 in the influent. Furthermore, Slipko et al. [19] studied the microbial community of AS
325 technology exposed to sub-inhibitory concentrations of CIP (0.1 – 100 µg/L), and their results
326 showed that the core of the microbiome was not significantly altered but rather adapted to
327 the antibiotic concentrations. Future studies would be focused on determining the correlation
328 and interplays between antibiotic addition and AR development in activated sludge.

329 In terms of ARB reductions, the values ranged from 0.7 to 3.6 log units during E1 and from
330 0.3 to 0.7 log units during E2. These lower performances, particularly in E2, suggest that
331 ARB and other AR elements (antibiotic and ARGs) may be persistent in the sewage sludge.
332 ARB, along with bacteria in general, tend to accumulate in the solid phase during AS

treatment due to the adsorption process [24,40]. In wastewater treatment, especially in biological systems, these contaminants are removed by physical processes such as filtration, sedimentation, and adsorption in the sludge [37,41]. Although ARB abundances were not detected in the stabilization and E1 stages which indicate high removal efficiencies, ARB may attach to and accumulate in the sludge biomass. For future research, it would be interesting to analyze the occurrence of ARB in sludge samples to determine the effects of antibiotic exposure on biomass. Murray et al. [42] investigated the presence of ARB and ARGs in sewage sludges and in crops fertilized with sewage sludge, reporting ARB abundances between 10^3 – 10^5 CFU/g·dry weight in sewage sludge. The occurrences of AR elements pose risk for the reuse of treated wastewater and treated sewage sludge from WWTPs in agriculture [43]. The spread of AR under such conditions could have detrimental effects on both the environment and human health [44,45].

Figure 5

4. Conclusions

The results of this study indicated that the combined antibiotic concentrations had a significant effect on the AS biomass in terms of performance and kinetic parameters. During E1, the COD and BOD_5 removals decreased to 42% and (-32%), respectively, and a variable trend was observed for OUR and SOUR reporting minimum values of below 20 $mgO_2/L \cdot d$ and 100 $mgO_2/gVSS \cdot d$, respectively. On the other hand, the AS biomass recovered during E1, achieving organic removal efficiencies close to 90% and OUR and SOUR values of 800 $mgO_2/L \cdot d$ or $mgO_2/gVSS \cdot d$. This recovery can be related to occurrence of ARB during E2 reporting abundances between 6.6 – 8.2 $x10^2$ CFU/mL. This result demonstrated that the exposures (E1 and E2) contributed to AR spreading into the reactor generating an adaptation of bacteria to selective pressure exerted by combined antibiotics.

CRediT authorship contribution statement

Burgos, J.: Investigation, Methodology, Writing- original draft; **Leiva. A.M.:** Validation, Conceptualization, Writing – review & editing, Data curation; **Gómez, G.:** Validation, Conceptualization; **Vidal, G.:** Validation, Conceptualization, Writing – review & editing, Supervision, Resources.

Declaration of Competing Interest

The authors declare that there are no competing financial interests on the publication of this article.

Data availability

Data are contained within the article and Supplementary Materials.

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Figure Captions

Figure 1. The experimental approach of this study (AMX: amoxicillin; CIP: ciprofloxacin; CTX: ceftriaxone; E1: exposure 1; E2: exposure 2; R1: reactor 1; R2: reactor 2; FE1: final effluent from reactor 1; FE2: final effluent from reactor 2; red point: sampling points).

Figure 2. Removal efficiencies of **a)** organic matter (chemical oxygen demand (COD) ■ and biological oxygen demand (BOD_5) ●), **b)** nitrogen forms (total nitrogen (TN) ▲ and ammonium nitrogen (NH_4^+-N) ◆) in reactor 1 (R1).

Figure 3. Principal component analysis (PCA) between reactor samples in stabilization (■, exposures 1 (E1, ■) and exposure 2 (E2, ■). The principal components 1 and 2 (PC1 and PC2) explained 37.6% and 19.6% of the total variance, respectively. The following symbols represent the different water samples: △ Influent; ○ exposure 1 (E1) and ◇ exposure 2. In this case,

Figure 4. a) Heterotrophic activity of reactor 1 (R1) biomass (oxygen uptake rate (OUR) ■ and specific oxygen uptake rate (SOUR) ●); **b)** Relation between sludge volumetric index (SVI) and food/microorganism ratio (F/M) where ■ corresponds to the biomass of R1 during the period of operation with normal feeding, ● R1 after exposure 1 (E1) and ◆ reactor 2 (R2) after exposure 2 (E2).

Figure 5. Abundances of antibiotic-resistant bacteria (ARB) during stabilization (■, exposure 1 (E1, ■) and exposure 2 (E2, ■) in water samples (I: influent; FE: final effluent). (amoxicillin: AMX; ciprofloxacin (CIP); ceftriaxone (CTX); colony forming unit (CFU)).

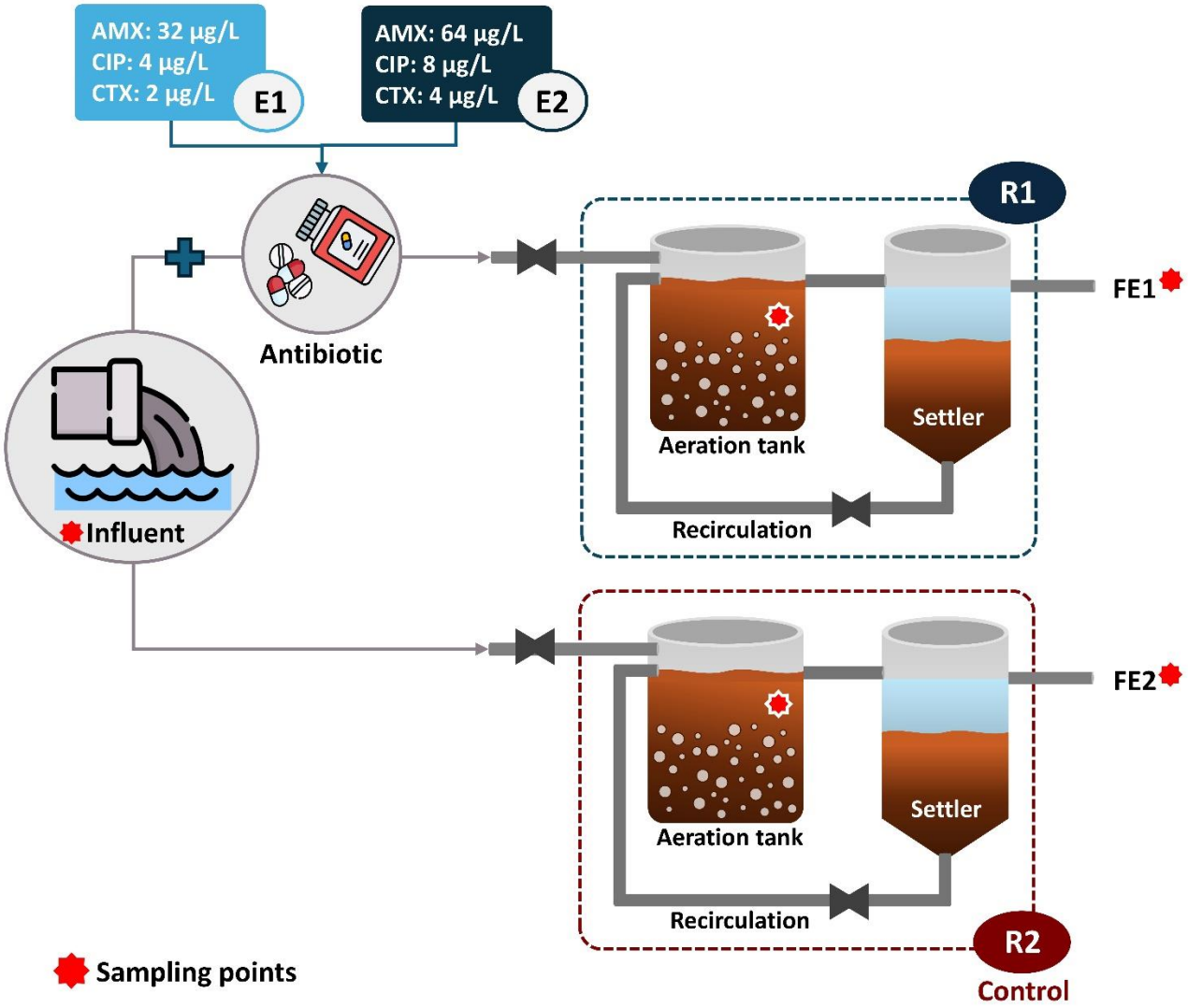
AMX: 32 µg/L
CIP: 4 µg/L
CTX: 2 µg/L
E1
AMX: 64 µg/L
CIP: 8 µg/L
CTX: 4 µg/L
E2
Antibiotic
Influent
R1
Aeration tank
Settler
Recirculation
FE1
FE2
Aeration tank
Settler
Recirculation
R2
Control
Sampling points

Figure 1

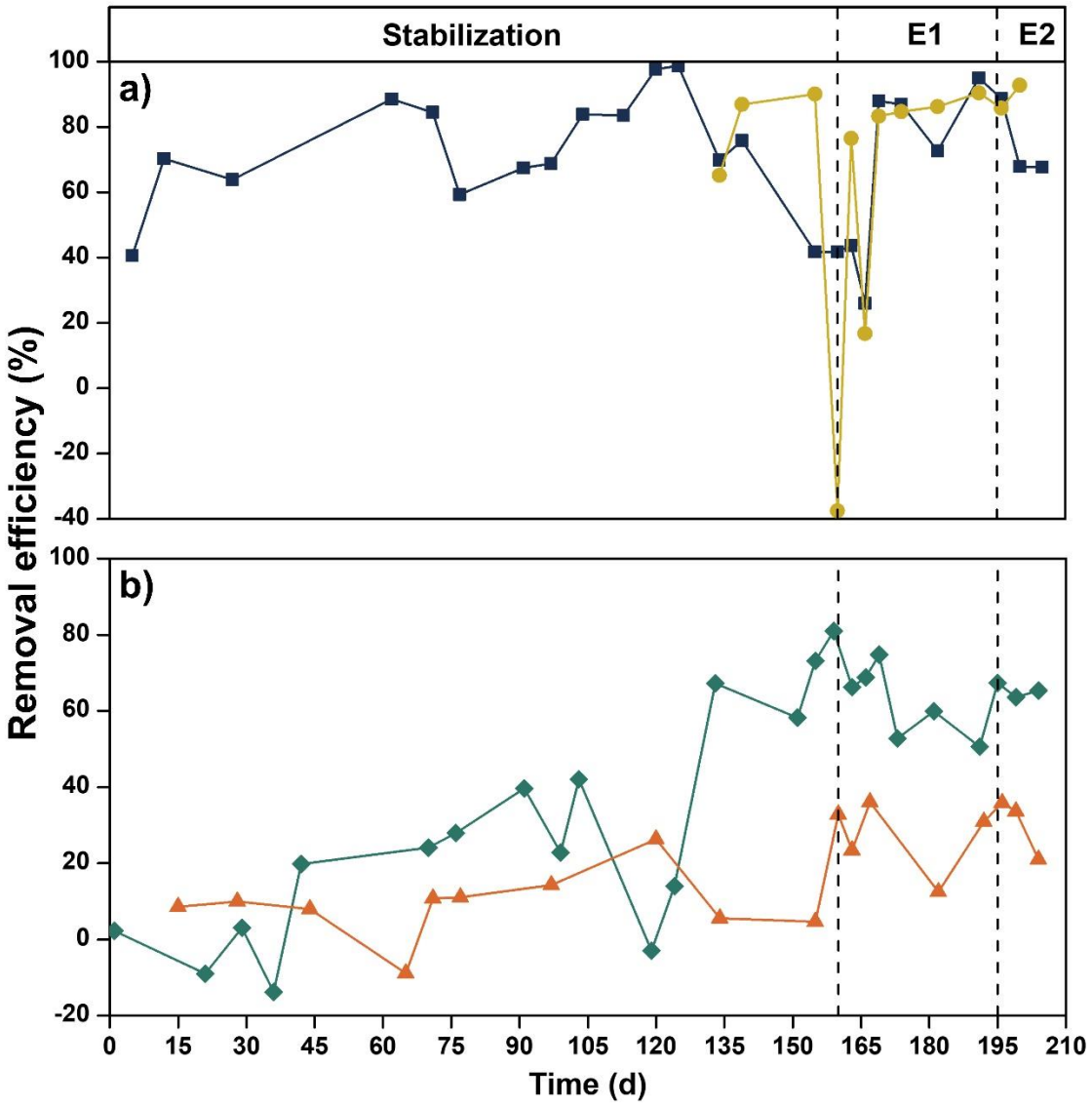
Stabilization
E1
E2
a)
b)
Removal efficiency (%)
Time (d)
100
80
60
40
20
0
-20
-40
0
15
30
45
60
75
90
105
120
135
150
165
180
195
210

Figure 2

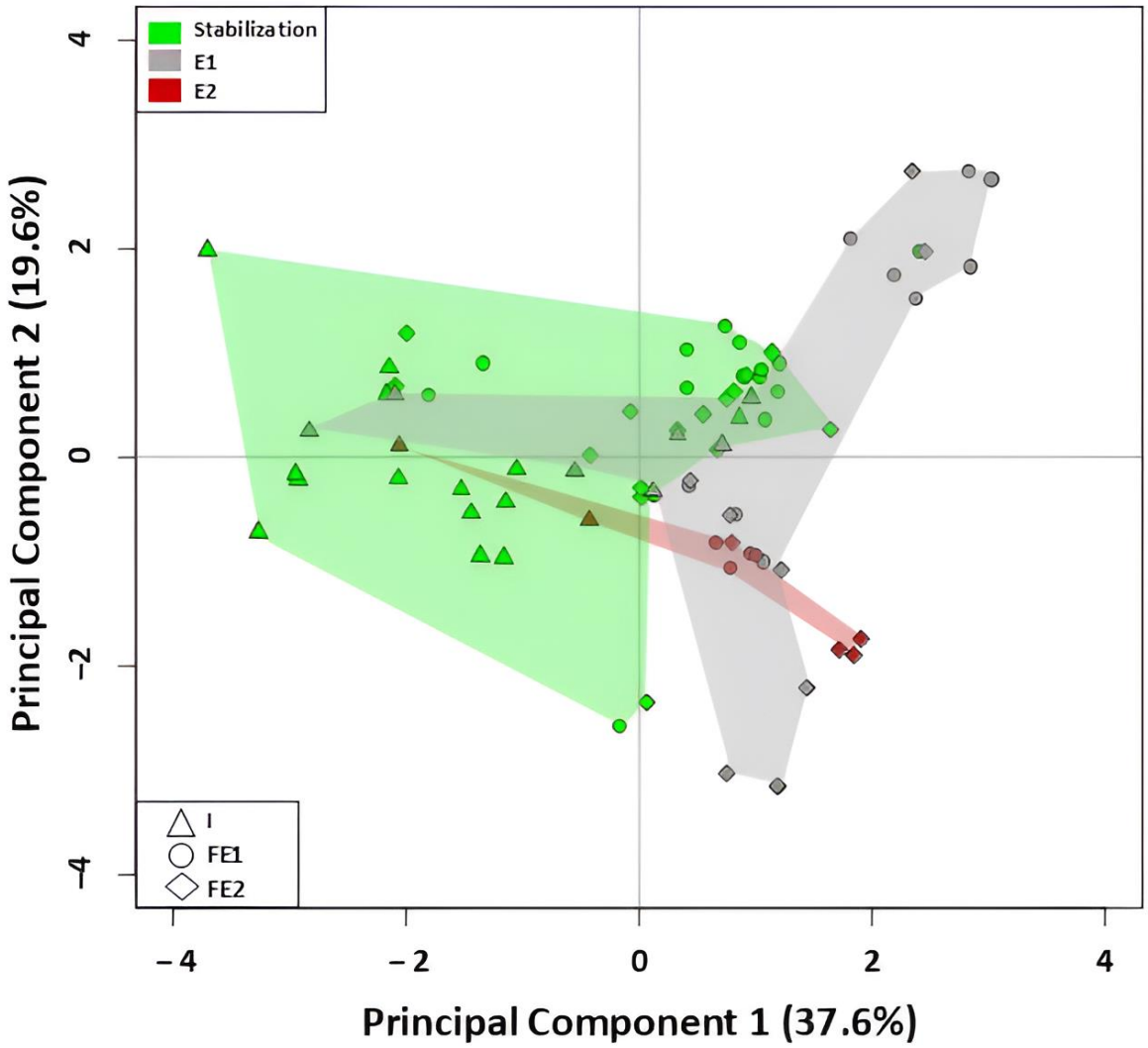
Stabilization
E1
E2
I
FE1
FE2
Principal Component 2 (19.6%)
Principal Component 1 (37.6%)
4
2
0
−2
−4
−4
−2
0
2
4

Figure 3

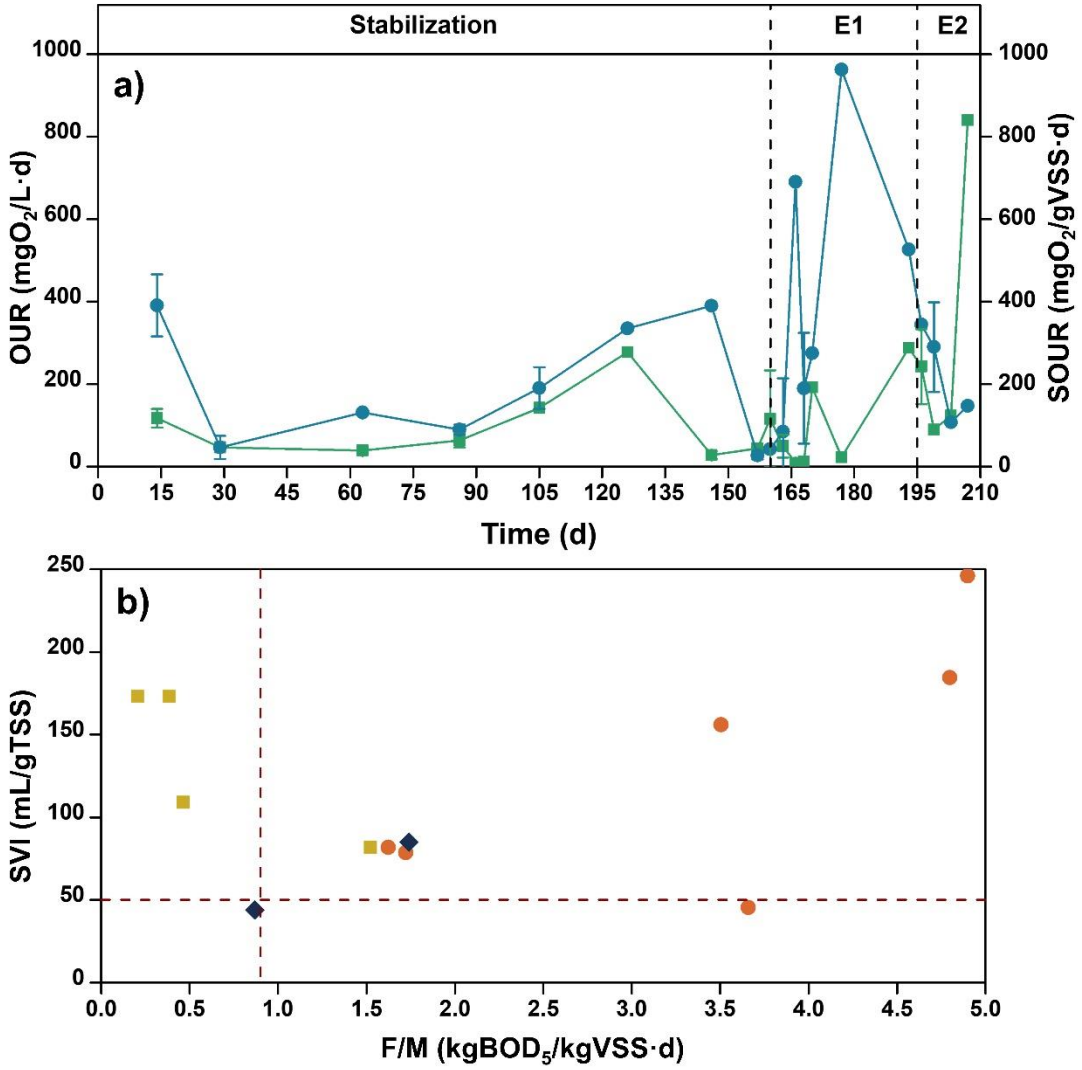
Stabilization
E1
E2
a)
OUR (mgO2/L·d)
SOUR (mgO2/gVSS·d)
Time (d)
b)
SVI (mL/gTSS)
F/M (kgBOD5/kgVSS·d)

Figure 4

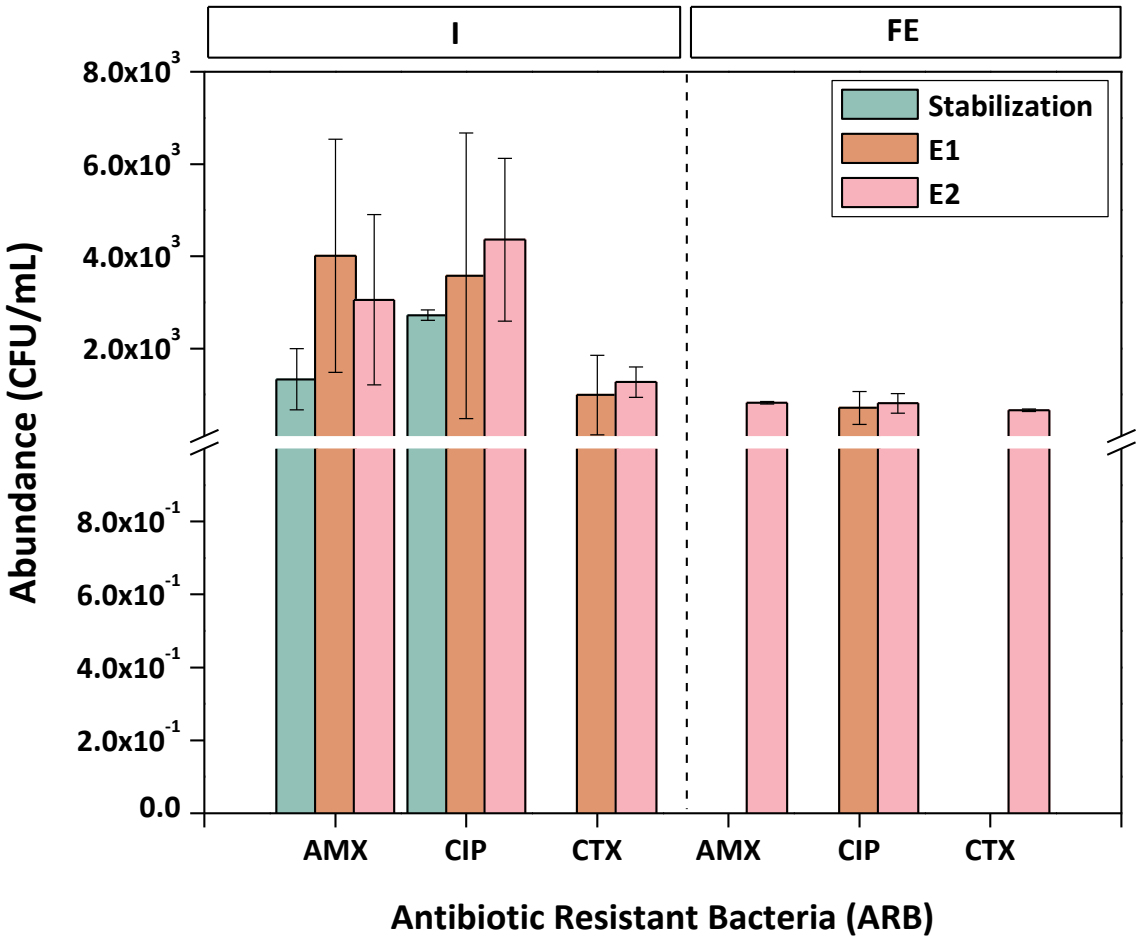
I
FE
Stabilization
E1
E2
8.0x10^3
6.0x10^3
4.0x10^3
2.0x10^3
8.0x10^-1
6.0x10^-1
4.0x10^-1
2.0x10^-1
0.0
Abundance (CFU/mL)
AMX
CIP
CTX
AMX
CIP
CTX
Antibiotic Resistant Bacteria (ARB)

Figure 5

Table Captions

Table 1. Physicochemical characterization of the influent during different stages of operational time.

Table 2. Biomass characterization of reactor 1 (R1) and reactor 2 (R2).

Table 4. Kinetic parameters of reactor 1 (R1) during the heterotrophic activity.

Parameters	Unit	Stabilization	E1	E2
pH	--	7.4 ± 0.3	7.2 ± 0.5	6.9 ± 0.2
T	°C	13.6 ± 3.7	21.0 ± 2.9	20.7 ± 0.9
EC	mS/cm	1.8 ± 0.2	1.5 ± 0.4	1.4 ± 0.5
ORP	mV	-183.2 ± 94.8	-127.7 ± 30.9	-218.0 ± 59.4
DO	mg/L	0.7 ± 0.4	3.0 ± 1.3	0.3 ± 0.1
Turbidity	NTU	188.6 ± 100.6	61.4 ± 14.5	54.6 ± 6.2
COD	mg/L	217.4 ± 131.1	96.2 ± 56.0	175.8 ± 54.1
BOD_5	mg/L	202.9 ± 49.3	75.5 ± 21.3	100.2 ± 23.3
PO_4^{3-}-P	mg/L	20.7 ± 8.6	24.7 ± 9.3	11.1 ± 0.2
NH_4^+-N	mg/L	114.5 ± 50.2	105.8 ± 46.9	123.6 ± 10.3
TN	mg/L	115.7 ± 28.7	112.4 ± 29.8	125.3 ± 21.2
TP	mg/L	21.2 ± 12.2	26.2 ± 12.3	15.2 ± 2.1

Notes: E1: exposure 1; E2: exposure 2; EC: electric conductivity; ORP: oxidation-reduction potential; T: temperature; DO: dissolved oxygen; COD: chemical oxygen demand; BOD_5: biological oxygen demand; TN: total nitrogen; TP: total phosphorus, NTU: nephelometric turbidity unit.

Table 1

Parameters	Unit	R1	R2
TSS	g/L	1.0 ± 0.1	1.5 ± 0.2
VSS	g/L	1.0 ± 0.2	1.5 ± 0.1
OUR	$mgO_2/L \cdot d$	144.3 ± 22.5	124.2 ± 30.2
SOUR	$mgO_2/gVSS \cdot d$	303.2 ± 75.0	160.6 ± 20.2
SVI	mL/gTSS	134.6 ± 27.4	184.0 ± 41.0

Notes: R1: reactor 1; R2: reactor 2; TSS: total suspended solids; VSS: volatile suspended solids; OUR: oxygen uptake rate; SOUR: specific oxygen uptake rate; SVI: sludge volumetric index

Table 2

Parameters	Unit	Stabilization		E1		E2	
		Range	Average ± SD	Range	Average ± SD	Range	Average ± SD
OUR_{end}	$mgO_2/L \cdot h$	5.3 - 20.9	10.6 ± 5.3	1.0 - 13.4	5.4 ± 4.1	6.5 - 12.3	10.7 ± 2.9
OUR_{exo}	$mgO_2/L \cdot h$	6.28 - 42.4	17.3 ± 12.0	1.5 - 22.4	8.7 ± 7.8	10.2 - 47.1	24.3 ± 16.0
OUR_{net}	$mgO_2/L \cdot h$	0.9 - 26.2	6.7 ± 7.7	0.2 - 12.0	3.3 ± 4.1	3.7 - 35.0	13.5 ± 14.6
SOUR	$mgO_2/gVSS \cdot d$	0.8 - 27.7	10.8 ± 9.0	3.5 - 181.8	38.1 ± 57.1	4.5 - 41.8	16.1 ± 17.4
OC	mgO_2/L	1.5 - 5.5	3.1 ± 1.3	0.6 - 4.6	2.1 ± 1.5	2.4 - 4.9	3.7 ± 1.0

Notes: OURend: endogenous oxygen uptake rate; OURexo: exogenous oxygen uptake rate; OURnet: net oxygen uptake rate; SOUR: specific oxygen uptake rate; OC: oxygen consumed; E1: exposure 1; E2: exposure 2; SD: standard deviation.

Table 3