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REMOVAL OF ESTROGENS BY ACTIVATED SLUDGE UNDER DIFFERENT CONDITIONS USING BATCH EXPERIMENTS

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Abstract - Wastewater treatment by deactivated and activated sludge was investigated to evaluate the removal of estrogens [estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2)] via adsorption and degradation. Different treatment conditions were used, including three mixed liquor volatile suspended solid (MLVSS) concentrations, three methanol concentrations (carbon source) and three types of aqueous media (water, synthetic solution, and supernatant). The E2 was degraded the fastest by the bacterial community. In all cases the removal rate increased when the initial MLVSS and methanol concentrations increased and when the macro- and micronutrients were present in the solution. In the experiments with deactivated sludge, the synthetic compound EE2 was more easily removed via sorption. The bacterial communities of the activated sludge were studied, which indicated a similarity of more than 75% between the different samples. A similarity of only 50% was found between the activated and deactivated sludges.
Keywords: Activated sludge; Estrogen removal; Bacterial community structure, E1, E2 and EE2.

INTRODUCTION

In aquatic environments, estrogens are micropollutants that have deleterious effects on aquatic organisms and water users. Conventional and current systems for wastewater treatment are not adequate for efficiently preventing these compounds from reaching the receiving water bodies. Currently, no federally mandated effluent discharge or sludge application standards for estrogens exist. According to Hecker and Hollert (2011), many efforts have focused on chemical risk assessments at the national and international levels. However, little has been discussed regarding the environmental risk assessments. Thus, it is possible that estrogens will be regulated in the future, but no guidelines have been suggested yet (Marti and Batista, 2014).

Many studies have focused on biological processes, such as activated sludge, for removing estrogens from wastewater. These studies have addressed the different conditions found in these processes, but the profiles of the bacterial communities that are found in sludge have rarely been reported. Li *et al.* (2005) found that the degradation of 17β-estradiol

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(E2) strongly depends on the analyte concentration, temperature, and mixed liquor volatile suspended solid (MLVSS) contents. Li *et al*. (2008) observed that the presence of different glucose concentrations as an additional carbon source impaired E2 degradation. Joss *et al*. (2004) compared the sludges from activated sludge and membrane bioreactor treatment systems and found that the sludge age and the size of the sludge flocs significantly influenced estrogen removal. Hashimoto and Murakami (2009) evaluated the presence of 17α-ethinylestradiol (EE2) in the liquid and solid phases of activated sludge tests and verified a 2 h lag phase and the complete removal and degradation of EE2 after 24 h. Furthermore, Chang *et al*. (2006) considered the degradation of estrone (E1) and E2 in short-term (72 h) tests and found that both E1 and E2 were removed (approximately 100%) when using initial concentrations of 100 and 1000 $\mu g\ L^{-1}$. Desmiarti and Li (2013) observed that E1 and E2 were completely degraded after 8 h in aerobic conditions. Moreover, E2 disappeared much faster than E1, which suggested that E1 was most likely more persistent than E2.

The main focus of most studies carried out in Brazil has been to verify the occurrence of these compounds in the aquatic environment (Kuster *et al*., 2009; Lopes *et al*., 2010; Montagner and Jardim, 2011; Moreira *et al*., 2009; Sodré *et al*., 2010; Ternes *et al*., 1999b) and to quantify the removal efficiencies of current sewage treatment systems (Brandt *et al*., 2013; Pessoa *et al*., 2014; Queiroz *et al*., 2012).

In this study, the simultaneous degradation of two natural estrogens, E1 and E2, and a synthetic estrogen, 17α-ethinylestradiol, EE2, was evaluated. The study was conducted in aerobic conditions and in a batch study using activated sludge samples under different conditions. In addition, the profiles of the bacterial communities that were present in each sludge sample were studied. The behavior of one deactivated sludge sample was studied regarding estrogen removal via adsorption. This research may help us to better understand the behavior of natural and synthetic estrogens in wastewater treatment plants (WWTPs), which is beneficial for determining the most efficient operational conditions for their removal and preventing their release into the environment.

MATERIALS AND METHODS

Solution Preparation

E1, E2, and EE2 were acquired from Sigma-Aldrich, Saint Louis, USA, with a purity of more than 98%. Stock solutions were prepared in high performance liquid chromatography (HPLC)-grade methanol at concentrations of 100 and 400 $mg\ L^{-1}$ and were stored at –20 °C for up to three months prior to use. The HPLC-grade methanol was used in all tests. The estrogen solutions without methanol were prepared by dissolving the estrogen compounds in milli-Q water at concentrations of 1.5 $mg\ L^{-1}$ and storing at 4 °C for 15 days.

A modified Dominic and Graham's (MDG) solution that contained 3.5 g of K_2HPO_4, 1.5 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.5 g of NaCl, 0.15 g of $MgSO_4\ 7H_2O$, and 1.0 mL of trace elements per liter with 0.005% (wt/vol) yeast extract (with trace element contents of 2.0 g of $NaHCO_3{\cdot}10H_2O$, 0.3 g of $MnSO_4{\cdot}4H_2O$, 0.2 g of $ZnSO_4{\cdot}7H_2O$, 0.02 g of $(NH_4)Mo_7O_{24}{\cdot}4H_2O$, 0.1 g of $CuSO_4{\cdot}5H_2O$, 0.5 g of $CoCl_2{\cdot}6H_2O$, 0.05 g of $CaCl_2{\cdot}2H_2O$, and 0.5 g of $FeSO_4{\cdot}7H_2O$ per liter) as a nutrient source was used as the synthetic medium (Yoshimoto *et al*., 2004). The supernatant medium consisted of the liquid that was removed from the activated sludge sample after sedimentation for 30 min.

Sludge Characterization

The activated sludge was collected directly from the aeration tank of a wastewater treatment plant (WWTP) that was located in Florianopolis, Santa Catarina, Brazil. This plant was considered representative in terms of flow and population, which were 22723 $m^3\ d^{-1}$ and 150000 inhabitants, respectively, because 100% of the wastewater was domestic. The WWTP treatment system consists of a biological selector and extended aeration activated sludge. A portion of the collected sludge was deactivated through sterilization using an autoclave at 120 °C for 30 min (as described by Chen and Hu (2010), Gusseme *et al*. (2009) and Li *et al*. (2005)).

Batch Experimental Protocol

To evaluate estrogen removal, kinetic tests were conducted using batches with 10 identical aerobic bioreactors (5 L volume). Air diffusers were installed inside of each reactor, and a pump was used to provide the oxygen that was necessary for bacterial maintenance. The reactors were fed once with a sufficient amount of activated sludge, which was collected at the WWTP, and with the corresponding medium (synthetic wastewater, water or Supernatant) to achieve the desired MLVSS contents (750 mL of working volume). The estrogens E1, E2, and EE2 were spiked simultaneously to achieve an initial

concentration of 100 μg L^{-1}. The highest methanol concentration in the systems represented 0.1% (v/v). The test conditions are shown in Table 1.

All of the assays were conducted at least twice in the dark, and each series lasted for 24 h. During this period, the operating conditions, such as temperature, pH, and oxygen, were held constant for all reactors. However, contact times of 0.5, 1, 4, 8, and 24 h were used. At the end of the set operating time, the aeration and agitation were switched off to allow for sedimentation. An aliquot (50 mL for 0.5 and 1 h; 100 mL for 4 h; and 200 mL for 8 and 24 h) of the resultant supernatant was subjected to solid phase extraction (SPE) using a commercial cartridge (see procedure below) for sample clean up and the analyte concentrations were determined using HPLC. Parallel blank assays were made to evaluate the possible degradation of the estrogens in the absence of sludge.

Table 1: Batch aerobic degradation experimental conditions.

Assays	Methanol (mg L^{-1})	MLVSS (mg L^{-1})	Medium	Type of sludge
01	750	3000	Synthetic	Deactivated
02	750	3000	Synthetic	Activated
03	750	1000	Synthetic	Activated
04	750	2000	Synthetic	Activated
05	750	2000	Water	Activated
06	750	2000	Supernatant	Activated
07	197.5	2000	Supernatant	Activated
08	0	2000	Supernatant	Activated

Analysis

Physicochemical Analysis

A spectrophotometer (Model DR/2010-Hach) and the corresponding kits (Test 'N Tube™ Vials) were used to determine the ammonium (NH_4^+-N) concentration according to the Salicylate Method (Hach). The gravimetric method was used to determine the MLVSS contents (APHA, 2005), and the pH was measured with a Thermo Scientific Orion pH meter. The temperature was measured with a digital thermometer, and a portable oximeter YSI-55 was used to measure the dissolved oxygen (DO) concentrations.

Estrogens

Solid Phase Extraction Procedure

This procedure is necessary for concentrating the estrogens in the samples, which permits the subsequent HPLC analyses. After the estrogen removal experiments, the samples were initially filtered using a glass fiber membrane with a 0.7 μm cutoff. Next, the pH of the filtered sample was adjusted to 3.0 with 6.0 mol L^{-1} HCl. Next, 6 mL of methanol and 6 mL of milli-Q water (pH = 3.0) were added to condition the cartridge (SPE SampliQ C18, 1000 mg, 6 mL, Agilent HP). Next, the sample was percolated through the cartridge (50 mL for 0.5 and 1 h; 100 mL for 4 h; and 200 mL for 8 and 24 h). Then, 5 mL of Milli-Q water was added to remove the impurities and the cartridge was dried under vacuum for 1 h. The analytes were eluted with 5 mL of acetonitrile. A rotary evaporator was used to evaporate the solvent at a temperature of less than 60 °C. The extract was reconstituted by adding 1 mL of methanol. Thus, the concentration factor depended on the sample volume initially used. Specifically, concentration factors of 50, 100 and 200x corresponded to volumes of 50, 100 and 200 mL.

HPLC Experimental Conditions and Validation

The chromatographic method was validated using analytical parameters, including the linearity, limit of detection (LD), limit of quantification (LQ), accuracy, and precision. The linearity was evaluated by injecting the samples for the standard curve in triplicate over ranges of 50-500 μg L^{-1} and 200-5000 μg L^{-1} prepared in methanol. The method precision was calculated using the coefficient of variation. The accuracy was assessed through recovery experiments that were performed in triplicate for three concentration levels, low (0.5 μg L^{-1}), medium (10 μg L^{-1}), and high (80 μg L^{-1}), with the acceptable values of 70-120% that were established by the Analyst Group of Pesticide Residues (GARP, 1999). The LD and LQ were obtained from the analytical curves (Equations (1) and (2). The compounds were quantified by external standardization.

$$LD = 3.3 \times SD\ S^{-1} \tag{1}$$

$$LQ = 10 \times SD\ S^{-1} \tag{2}$$

Here, SD is the standard deviation of the linear coefficient, and S is the slope of the analytical curve.

HPLC analyses were made using a Varian chromatograph, model 920 LC, with a diode array detector (DAD) and a 5 μm C18 column of 250 × 4.6 mm (Varian). The chromatographic conditions that were established to determine the concentrations of the estrogens were as follows: flow rate = 0.8 mL min^{-1},

injection volume = 50 µL; detection = 197 nm DAD; column temperature = 30 ± 2 °C. In addition, the gradient elution system used acetonitrile (ACN) and water (H_2O) as solvents. The proportions of solvent were varied as follows: an initial H_2O:ACN condition of 90:10, the H_2O:ACN ratio was linearly changed to 30:70 over 26 min, the proportion of H_2O was decreased to zero over 5 min, the ACN-only conditions were maintained for 6 min (column cleaning), and the H_2O:ACN ratio was linearly changed to 90:10 over 3 min and then held at 90:10 over 7 min (column balancing).

Bacterial Community

The bacterial community structure contained in each sludge sample was characterized by nucleic acid extraction using a commercial kit (Power Soil, Mobio) following the manufacturer's protocol. The gene encoding the 16S ribosomal RNA was amplified by a polymerase chain reaction (PCR) using the F968 and R1401 (GC - clamp) (Evans *et al*., 2004; Li *et al*., 2006) primers and reagents at the optimized quantities recommended by the manufacturer of the taq DNA polymerase (code D4545, Sigma). Amplification was performed using a thermocycler (Eppendorf Mastercycler) under the conditions adopted by Evans *et al*. (2004). The PCR product was subjected to denaturing gradient gel electrophoresis (DGGE), and the gel was prepared with 6% polyacrylamide with a denaturing gradient of 25-60%. Electrophoresis was performed in 7 L of the 1X Tris-Acetate-EDTA (TAE) buffer at 130 V and 60 °C for 270 min. Next, the gel was stained using the Gel Red commercial solution for 50 min before viewing under ultraviolet (UV) light and capturing with an image capturing system. Cluster analysis, which was displayed as a dendrogram, was performed using Gel Compar II Version 6.5.

RESULTS AND DISCUSSION

Validation Parameters for the HPLC Method

Table 2 shows the data for the linearity, LD, LQ, accuracy, and precision. The LD, LQ, precision, and accuracy were within the ranges that are considered acceptable in the literature (Ribani *et al*., 2004). In addition, the results displayed excellent linearity. Compounds E1 and E2 displayed correlation coefficients greater than 0.999, which indicated that the regression line fit the data well (Ribani *et al*., 2004). Moreover, the limits shown in Table 2 do not consider the concentration factors of the samples; that is, all initial sample volumes (50, 100 and 200 mL) were concentrated to a final volume of 1 mL for injection. Therefore, the obtained LQ and LD values must be divided by the concentration factor to be considered. The LQ to E2, EE2 and E1 were 0.16, 0.13 and 0.08 µg L^{-1}, respectively, when 200 mL of solution was used.

Factors Affecting Estrogen Removal

The experiment to study the removal of the estrogens by sludge was designed based on one and seven assays with deactivated and activated sludge, respectively (as shown in Table 1). Assay 01 was used to evaluate the sorption abilities of the estrogens by the deactivated sludge in the absence of bacteria. The other assays were made to evaluate the influences of three factors on the biodegradation of the estrogens, the MLVSS contents (assays 02, 03 and 04), the micronutrient contents provided (or not) in the aqueous medium (assays 04, 05 and 06) and the methanol concentrations that were added as a carbon source (assays 06, 07 and 08 (without methanol)). Water was used to evaluate the capacities of the bacteria for

Table 2: Validation parameters for the method used to analyze the selected estrogens in different working concentrations.

Compound	Correlation coefficient (r)	LRC (µg L^{-1})	LD (µg L^{-1})	LQ (µg L^{-1})	Accuracy (%)[a]	Precision (%)[a]
E2	0.99907	50-500	9.6	32.0	107	14.6
EE2	0.99896		7.7	25.8	85	7.7
E1	0.99917		5.0	16.8	87	8.0
E2	0.9993	200-5000	24.9	83.0		
EE2	0.99878		24.4	81.2		
E1	0.99975		12.4	41.2		

LRC = linear concentration range; LD = limit of detection; LQ = limit of quantification.
[a]Average for the three concentration levels (0.5, 10, and 80 µg L^{-1}).

degrading the estrogens in the absence of nutrients, such as ammonium, that were contained in the synthetic medium.

The results obtained in the parallel blank assay, with the synthetic wastewater and the hormones that were dissolved in methanol without MLVSS, showed mean concentrations and standard deviations of 82.9 μg L^{-1} and 5.4 for E2, 84.2 μg L^{-1} and 4.31 for EE2 and 94.01 μg L^{-1} and 4.32 for E1 throughout the test period and for all studied periods. This difference is due to the recovery of the extraction process, which indicated that no estrogen degradation resulted from the medium or methanol.

Activated and Deactivated Sludge

To verify the contributions of adsorption to the removal of estrogens, an assay with deactivated sludge was conducted (Table 1, assay 01 and Figure 1a). The EE2 concentration decreased with time more than the E1 and E2 concentrations (94% in 1 h). This fact potentially resulted from the greater hydrophobicity of EE2 ($LogK_{ow}$ = 4.1) compared to the natural E1 and E2 estrogens ($LogK_{ow}$ = 3.1 and 3.4 for E1 and E2, respectively). In addition, this result agrees with the results of studies that were conducted by Urase and Kikuta (2005), Ren *et al*. (2007) and Zhang *et al*. (2012), in which 60% of EE2 estrogen was removed during the first 20 min, 98% was removed during the first 10 min and more than 80% in the first 1h, respectively.

In the same assay with the deactivated sludge, the E2 and E1 concentrations decreased by approximately 81.5 and 76.7%, respectively, in 24 h. These results are in agreement with those of Ren *et al*. (2007) (93.6% for E2 and 98.0% for E1 removal) and Shi *et al*. (2013) (60% E1 removal within 8 h). In contrast, the E2 removal was higher than that obtained by Li *et al*. (2005), who found a maximum E2 removal of 10% after 20 h using 30 μg L^{-1} of E2 and a MLVSS content of 1750 mg L^{-1}.

The sorbent specific characteristics of the sludge and the experimental conditions largely affected the estrogen removal results, corresponding with the results of Chen and Hu (2010), Racz and Goel (2010) and Silva *et al*. (2012). This finding could explain the sorption variability that was observed among the different studies.

The amounts of estrogen that were adsorbed at equilibrium on the deactivated sludge can be calculated by using Equation (3) (Ren *et al*. 2007).

$$q = (C_0 - C_e) V M^{-1} \quad (3)$$

Here, q is the amounts of estrogen adsorbed on the deactivated sludge (μg g^{-1} VSS), C_e is the equilibrium estrogen concentration in the aqueous phase (μg L^{-1}), C_0 is the initial estrogen concentration of the solution (μg L^{-1}), V is the volume of the solution (L) and M is the sludge weight (g VSS).

Using this equation, we obtained estrogen concentrations of 27.33, 25.67 and 32.67 μg g^{-1} for E2, E1 and EE2, respectively (Table 3). These results confirmed that more of the synthetic hormone was adsorbed than the other estrogens during the first 24 h. This trend was also observed by Ren *et al*. (2007).

Table 3: Adsorption parameters for calculating the estrogen concentrations in the deactivated sludge.

	E2	E1	EE2
C_0 (μg L^{-1})	100	100	100
C_e (μg L^{-1})	18	23	2
Time (h)	24	24	24
M (g)	2.25	2.25	2.25
V (L)	0.75	0.75	0.75
q (μg g^{-1} VSS)	27.33	25.67	32.67

The results of assays 01 and 02 were compared because the only difference between them was the sludge. In assay 02 (Figure 1b), E2 and E1 were no longer detected after 4 and 8 h, respectively.

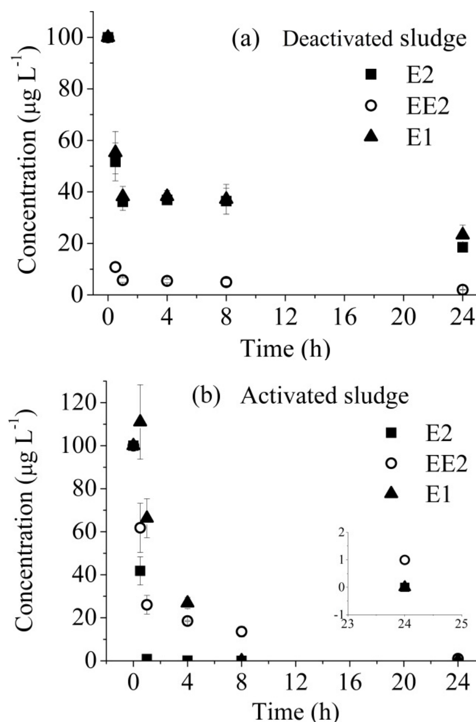

Figure 1: The removal of estrogens with (a) activated sludge and (b) deactivated sludge (Error bars give the standard deviation ± SD). Insert: estrogen concentrations over 24 h.

However, synthetic EE2 was observed after 24 h of testing (0.99 μg L^{-1}) despite its high removal (99%). This result contradicted the results obtained by Ternes *et al*. (1999a), who evaluated estrogen degradation in aerobic batch experiments with initial concentrations of 1000 and 1 μg L^{-1} and only observed a 20% decrease in the EE2 concentration after 48 and 24 h.

The removal of hormones from the wastewater in an activated sludge system mainly involves adsorption and degradation processes (Racz and Goel, 2010; Silva *et al*. 2012). Generally, after fast initial adsorption on the sludge, biodegradation will reduce the estrogen levels in the aqueous and solid phases (Desmiarti and Li, 2013; Marti and Batista, 2014). According to Racz and Goel (2010), immediate estrogen losses in activated sludge, which occurred in our study, can largely be attributed to sorption.

The E1 and E2 removals were greater in assay 02, which contained activated sludge, than in assay 01, which contained deactivated sludge (Figure 1). This result may indicate the existence of a bacterial degradation mechanism, which most likely coexists with an adsorption mechanism in the case of the natural estrogens. In contrast, the EE2 removal was slower in the activated sludge than in the deactivated sludge. This finding suggests that the bacterial community had more trouble degrading the synthetic estrogen EE2. Next, adsorption would be the primary mechanism of EE2 removal by the sludge.

When the microorganisms were present, the combined effects of adsorption and degradation were observed, mainly involving the natural hormones, where this effect was more pronounced. The adsorption and degradation processes of the natural hormones occurred simultaneously. However, the removal of EE2 was slower due to the higher affinity of the microorganisms for E1 and E2 than for EE2. However, after 24 h, the EE2 removal was the highest when activated sludge was used, confirming the occurrence of biodegradation and adsorption processes. Thus, the bacteria begin to degrade EE2 when the amount of easily assimilated hormones is already low.

The high EE2 removal obtained for the deactivated sludge could be explained by considering several aspects. According to Yi and Harper (2007), changes in the biomass particle size (e.g., porosity, density, available surface area, and the protein and carbohydrate contents of the flocs) affect the K_d (partitioning coefficients for sorption). Thus, the authors concluded that this factor could directly affect the way in which the adsorption process is conducted.

Furthermore, the deactivation process was conducted in an autoclave, which potentially modified the physical properties of the sludge flocs and could significantly affect their adsorption capacity. In the study carried out by Bougrier *et al*. (2008), the changes in the characteristics of five different activated sludge samples were evaluated after thermal treatment at between 90 and 210 °C. The authors found that the concentrations of the solid particles decreased and that more mineral particles remained. Solids, proteins, carbohydrates and lipids were solubilized and changes in their concentrations were observed. Heat treatment promotes changes in the physical characteristics of the sludge, affecting its viscosity, sedimentation and filterability.

If this research is applied to a continuous flow reactor, the results and suggestions provided by Dialynas and Diamadopoulos (2012) should be considered. Based on their results, the amounts of each compound that were removed by adsorption resulted from their adsorption to freshly grown biomass, which was equal to the amount wasted per day to maintain a constant level of suspended solids in the mixed liquor of the reactor. It is important to conduct new research for full-scale application and to ensure that the sludge removed from the wastewater treatment plants with adsorbed compounds is properly treated.

The results obtained by Xu *et al*. (2008) show that the EE2 sorption process is spontaneous ($\Delta G<0$). In addition, these authors suggested that these results could be directly applied to full-scale wastewater treatment plants because the concentrations found in the raw wastewater were much lower than those used in their study or in our study.

Xu *et al*. (2008) and Ren *et al*. (2007) observed that EE2 sorption involves physical sorption as the dominant mechanism in combination with a relatively low level of chemical reactions. Therefore, the compounds could be desorbed from the sludge to the effluent (reversible process). In addition, these authors observed that the desorption process was much slower than the adsorption process. Desorption was not detected in this study under the adopted conditions.

Assays 02 to 08, which contained activated sludge, had estrogen reductions of at least 65% in all cases within 24 h of contact time. In the seven assays, the E2 concentration decreased significantly during the first few minutes of the test and by more than 98.5% within 1 h. These results agree with the results that were obtained by Chang *et al*. (2006), in which an E2 removal of 50% was observed after only 30 min, with complete removal after 10 h for both tested concentrations (100 and 1000 μg L^{-1}). Similarly, Desmiarti and Li (2013) observed that E2 decreased rapidly by up to 90% after 30 min and disappeared

completely after 2 h (LD = 0.01 μg L^{-1} for E2 and E1, liquid chromatography/mass spectrometry (LC/MS)).

The high E2 removal ability of the activated sludge potentially resulted from various factors, including biodegradation and/or sorption mechanisms. Thus, Li *et al*. (2008) considered that this estrogen is strongly adsorbed on the activated sludge particles, while other authors indicated that E2 is rapidly oxidized to E1 (Combalbert and Hernandez-Raquet, 2010; Desmiarti and Li, 2013; Joss *et al*., 2004).

MLVSS Contents

The treatment of E2 by activated sludge (assays 02, 03 and 04 in Table 1) was very effective, with more than 50 and 98.5% of estrogen removal after 30 min and 1 h, respectively, for the three evaluated MLVSS contents (1000, 2000, and 3000 mg L^{-1}). According to Chen and Hu (2009) and Li *et al*. (2005), the degradation of E2 increases as the MLVSS content increases, even if the maximum E2 concentration is significantly lower than the organic compound concentration found in the sewers. This result occurs because the presence of more microorganisms in the activated sludge can result in more effective degradation of the E2. In this study, the E2 removal was very fast, even when using the lowest MLVSS content. This result suggested contributions from the bacterial degradation, oxidation and adsorption mechanisms (as above discussed). Thus, in the assay with 1000 mg L^{-1} of MLVSS, the concentration of E1 increased after 1 h of testing, likely due to the oxidation of E2 to E1 (Figure 2a). The low MLVSS content in the system potentially contributed to the slow deterioration of E1. This low MLVSS content was not favorable for the degradation of EE2 (Figure 2b). Thus, only 76% of the EE2 was removed after 24 h. The best results were obtained for the highest MLVSS (2000 and 3000 mg L^{-1}) content, which potentially had the highest bacterial populations and a greater number of sorption sites.

Medium: Synthetic Wastewater, Water and the Supernatant Solution

Assays 04, 05 and 06 in Table 1 were conducted to compare the effects of the carbon source (supernatant) and other nutrients (synthetic solution) in the medium on estrogen removal. The initial ammonium (NH_4^+-N) concentration in the synthetic medium was 47 mg L^{-1}, but was less than 0.2 mg L^{-1} in the supernatant and water.

The E2 removal was not affected by the different media that were present in the system. A very high removal was observed during the first hour of the test, during which more than 98.5% of the E2 was removed from the aqueous phase.

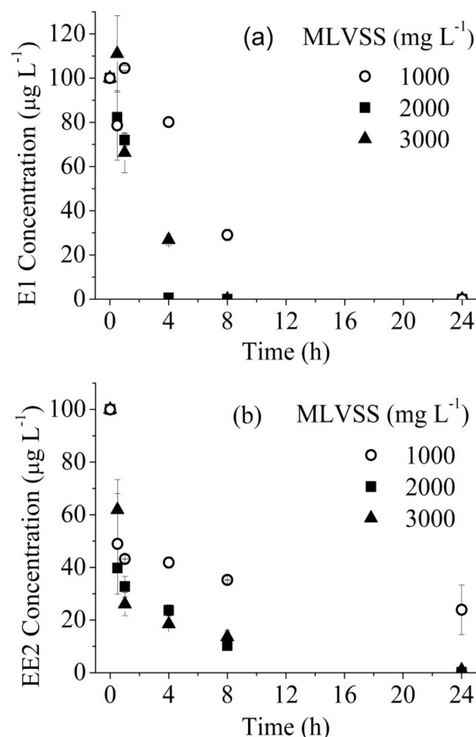

Figure 2: Degradation of (a) E1 and (b) EE2 for the different MLVSS contents (Error bars give the standard deviation ± SD).

The use of synthetic medium allowed for further degradation of E1 (Figure 3a) and EE2 (Figure 3b) relative to the water and supernatant, which indicated that the presence of ammonium accelerates the estrogen removal process. Thus, after 4 h, the E1 compound was no longer detected when the synthetic wastewater was used. However, the removal reached less than 60% during this period when water was used as a medium in the system. Within 8 h, the values obtained in the chromatographic analyses for determining the E1 contents were below the LQ of the method for all of the tested media. Regarding the EE2 compound, the effect of ammonium was less pronounced, potentially due to the lower ability of the bacteria to degrade the synthetic estrogen. However, this effect was observed and a higher removal was achieved after 4 h when the synthetic wastewater was present. After 24 h of testing, the removal of this compound was more than 89% in all of the tested media, reaching 99% in the presence of the synthetic wastewater.

The presence of methanol in the assays potentially favored high estrogen removal through come-

tabolism. This process is observed when bacteria use their existing enzymes to degrade steroidal hormones (Hamid and Eskicioglu, 2012; Yu *et al*., 2013). However, the positive effect of methanol was the same in the three studied cases because its concentration was held constant (750 mg L^{-1}), which was done to compare the effects of the three different media.

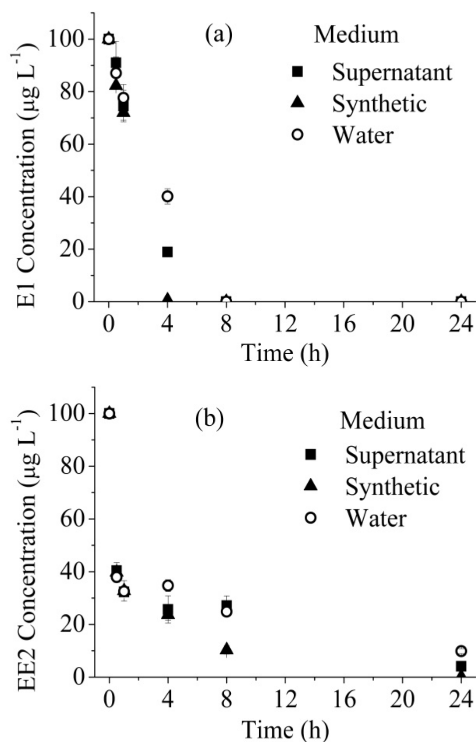

Figure 3: Degradation of (a) E1 and (b) EE2 in the different mediums (Error bars give the standard deviation ± SD).

The different media considerably affected the abilities of the microorganisms for degrading estrogens, mainly due to the presence of nutrients that are capable of promoting microbial growth and maintenance. Thus, the best results were obtained using the synthetic medium, which was rich in ammonium, followed by the supernatant. Water resulted in the worst estrogen removal.

Methanol Concentration

The influence of the methanol concentration as an additional carbon source during estrogen treatment by activated sludge was evaluated using assays 06, 07 and 08 (in Table 1). Methanol was not toxic to the microorganisms under the evaluated conditions, which corresponded with previous results (Estrada-Arriaga and Mijaylova, 2011). E2 was readily degraded, with 78-97% of the E2 removed during the first 30 min in all three test conditions.

The E1 and EE2 estrogens were removed more easily when the highest methanol concentration was used (Figure 4). Thus, when 750 mg L^{-1} of methanol was used, the removal of E1 was greater than 80% in 4 h (Figure 4a). However, in the absence of methanol, less than 45% removal occurred within 4 h for E1. After 8 h, the E1 removal reached 82% in the absence of methanol and E1 was no longer detected in the solution when methanol was added.

The removal of EE2 was not satisfactory when methanol was not present in the solution (Figure 4b). These results were in contrast with the behavior of E1 (Figure 4a), which indicated the lower ability of the bacterial community for degrading the synthetic estrogen. In addition, because these tests were conducted with supernatant media with low ammonium contents (< 0.2 mg L^{-1}), this finding potentially resulted from the lack of compounds that facilitate the removal of the synthetic hormone by cometabolism. The greater EE2 degradation that was observed when the methanol concentration was increased was potentially caused by cometabolism, as suggested by Larcher and Yargeau (2013).

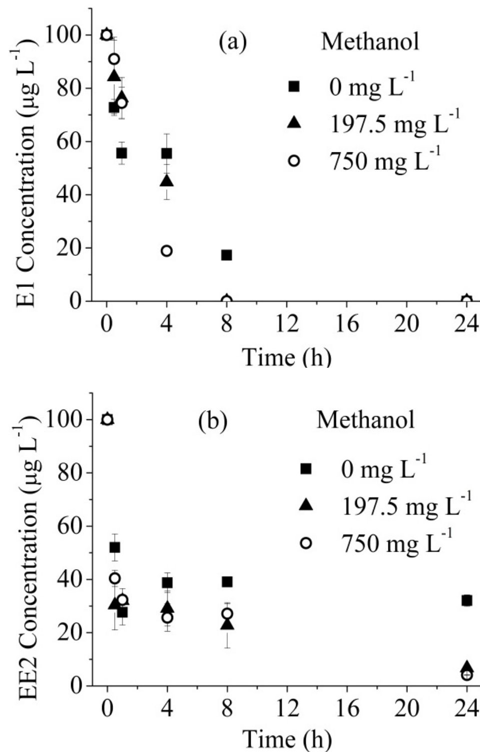

Figure 4: Degradation of (a) E1 and (b) EE2 under different methanol concentrations (Error bars give the standard deviation ± SD).

The Profiles of the Bacterial Community Structures that Were Present in Each Sludge Sample

The bacterial community present in each sludge sample was evaluated previously using the assays in Table 1 before adding the medium and the hormones dissolved in methanol. The samples were collected at the same point of the WWTP on different days. Figure 5 presents images from the DGGE analysis and dendrogram, which represent the cluster analysis. The similarity results of the activated sludge samples (Figure 5, samples 02-08) indicated that only slight changes occurred in the microbial community with time, which were most likely promoted by variations in the influent water quality, according to Miura *et al*. (2007) and Fernandes *et al*. (2013). Therefore, the microbial community structure did not change considerably on different days. Thus, these results indicate that the microbial community structure did not affect the variations in estrogen removal between the samples.

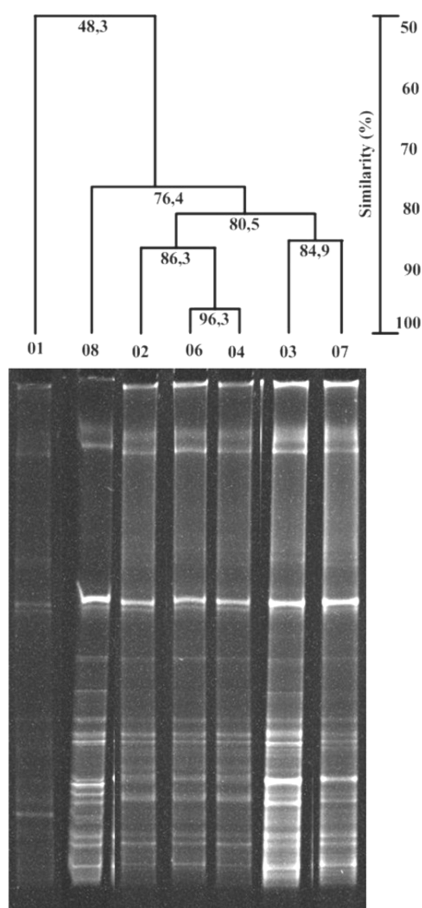

Figure 5: DGGE gel banding profile of the microbial communities. The numbers represent the assays in Table 1. Assays 03 and 05 used the same sample.

The different estrogen degradation rates that were obtained here indicate that the main factors affecting the abilities of the microbial population to degrade estrogen are the MLVSS contents, the media, and the methanol concentration present in the evaluated systems.

A comparison of the bacterial communities that are present in the activated (Figure 5, samples 02-08) and deactivated sludge samples (Figure 5, sample 01) indicated that the percentage of similarity among these samples was less than 50% after deactivation. The deactivation process modified the bacterial community structure in the sludge. The weaker lines (Figure 5 sample 01) indicate which part of the genetic material had been destroyed.

This research showed that microorganisms easily adapted to the presence of estrogens. The microorganisms could rapidly grow and effectively contribute to the hormone degradation. Thus, estrogen biodegrading bacteria are present in conventional systems with activated sludge. This result was also observed in the study conducted by Bagnall *et al*. (2012), in which the estrogen degradation was achieved using non specialized organisms within 24 h.

CONCLUSIONS

This study considered the simultaneous degradation of identical concentrations of E1, E2 (natural estrogens), and EE2 (synthetic estrogen). The E2 concentration was rapidly reduced by the activated sludge under all of the conditions considered. In addition, oxidation of E2 to E1 was observed. When deactivated sludge was used, EE2 had the highest removal, potentially due to the sorption of EE2 in the sludge. E1 and EE2 were easily removed from the liquid phase when high MLVSS contents and methanol concentrations were present and when the synthetic medium that was rich in ammonium was used. The activated sludge samples had similar microbial communities. Autoclaving was used to create deactivated sludge, which resulted in a similarity of less than 50% with respect to the activated sludge. Aerobic biodegradation that occurs in a conventional system, such as activated sludge, is an important mechanism for attenuating hormones from domestic wastewater (mainly natural hormones). The efficiency of this mechanism depends on the operational conditions, which are favored in the presence of additional carbon and ammonium sources. In addition, the adsorption of the estrogens on the sludge may contribute to their removal. The removal of the synthetic estrogen, EE2, was more difficult, but also

occurred by sorption in the sludge and/or by cometabolism.

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NOMENCLATURE

ACN	Acetonitrile
DAD	Diode Array Detector
DGGE	Denaturing Gradient Gel Electrophoresis
DO	Dissolved Oxygen
E1	Estrone
E2	17β-estradiol
EE2	17α-ethinylestradiol
HPLC	High Performance Liquid Chromatography
LC/MS	Liquid Chromatography/Mass Spectrometry
LD	Limit of Detection
LQ	Limit of Quantification
LRC	Linear Concentration Range
MDG	Modified Dominic and Graham's
MLVSS	Mixed Liquor Volatile Suspended Solid
PCR	Polymerase Chain Reaction
SD	Standard Deviation
SPE	Solid Phase Extraction
TAE	Buffer Tris-Acetate-EDTA
UV	Ultraviolet
VSS	Volatile Suspended Solid
WWTPs	Wastewater Treatment Plants

Symbols and Units

C_0	Initial estrogen concentration of the solution (μg L^{-1})
C_e	Equilibrium estrogen concentration in the aqueous phase (μg L^{-1})
K_d	Partitioning coefficients for sorption
K_{ow}	Octanol/water partition coefficient
M	Sludge weight (g VSS)
q	Amounts of estrogen adsorbed (μg g^{-1} VSS)
V	Volume of the solution (L)

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