Comparison of estrogen compounds removal efficiency in sample and alternating anoxic/aerobic activated sludge process

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Abstract: *The occurrence and the removal efficiency of natural estrogens, estrone (E1), 17β-estradiol (E2) and estriol (E3), as well as a synthetic estrogen, 17α-ethinylestradiol (EE2), were investigated in two sewage treatment plants (STPs) employing different activated sludge processes in Tunisia, by monitoring their concentrations in dissolved wastewater. The analytes were extracted by solid-phase extraction and then analyzed by gas chromatography coupled with mass spectrometry (GC-MS). Results indicate that natural and synthetic estrogens were detected in the STP influent samples. The concentrations of natural and synthetic estrogens (except E1) detected in the effluents of alternating activated sludge process were generally lower than the limit of detection. On the other hand, all estrogens were not totally removed through the simple activated sludge process; as a result, they were detected in effluent samples. E3 showed the highest concentration in the influent samples at 98 ng L^{-1} in the alternating anoxic/aerobic activated sludge process, and at 102 ng L^{-1} in the simple activated sludge. Two different types of diurnal variation of estrogens discharge were identified. The two STPs had approximately similar daily loads of estrogens in influents at 112.7 μg d^{-1} and 109.58 μg d^{-1}, respectively. The distribution of estrogens in the STPs is affected by their metabolisms in human body and their transition through biological treatment processes. Removal efficiency varied among STPs; high removal of estrogens (84-97%) with the exception of E1 (80%), were generally achieved in alternating anoxic/aerobic activated sludge process with a high hydraulic retention time (40 h) and treatment stages for removal of nitrogen. E1 showed the lowest removal of all estrogens in the two STPs. This could be caused by conversion of E2 to E1 in the treatment process, or by cleavage of estrogen conjugates. On the other hand, E3 showed the highest removal of all estrogens both in alternating anoxic/aerobic and simple activated sludge processes at 97.5% and 85.5%, respectively). EE2, which is particularly sensitive to human consumption as the oral contraceptive, were removed by approximately 77.5%) in the simple activated sludge process. Filamentous bacteria were less effective in removal estrogens than protozoa.*
Keywords: *sewage treatment plants, estrogens, alternating anoxic/aerobic activated sludge, hydraulic retention time, extraction, gas chromatography-mass spectrometry, microscopic examination*

I.   Introduction

The growing population, urbanization and modernization have led to the release of many organic pollutants into the environment. In recent years, the presence of endocrine-disrupting chemicals (EDCs) in the environment has been a major concern worldwide because they may alter the normal hormone functions as well as physiological status in wildlife and humans (Mills and Chichester, 2005; Khanal et al., 2006). Various types of natural and synthetic chemical compounds including estrogens have been identified as EDCs. The estrogens of natural origin include 17β-estradiol (E2) and its main metabolites (i.e., estrone (E1) and estriol (E3)), have been found to alter gonad differentiation and development, decrease fertility (Safe et al., 2000; Jobling et al., 2003) and sex ratio (Mackenzie et al.,2003), impair spermatogenesis (Lee et al., 2005), gonadal dysgenesis (Fort et al., 2004; Hecker et al., 2005), hermaphroditism (Hayes et al., 2003) and cause feminization (Levy et al., 2004; Kloas et al., 2006). In addition, 17α-ethinylestradiol (EE2) which is widely used as an oral contraceptive, can bind to the estrogen receptor and misregulate or interfere with normal biological responses. This synthetic hormone has caused feminization in several fish species (Kwak et al., 2001; Mills and Chichester, 2005), at concentrations as low as 0.1 ng L^{-1} (Purdom et al., 1994). Many studies have shown that municipal sewage treatment plants (STPs) are a significant point source of EDCs released into the environment (Lee et al., 2002; Stasinakis et al., 2008; Zhang and Zhou, 2008; Zhou et al., 2009). EDCs discharge can exhibit seasonal, weekly and diurnal variation (Joss et al., 2005; Graver et al., 2011; Nie et al., 2011).

Previous research showed that estrogenic hormones have been detected in influents and effluents of STP in many countries (Baronti et al., 2000; Jeannot et al., 2002; Stasinakis et al., 2010) and surface water (Boyd et al., 2004; kim et al., 2007; Stasinakis et al.,2008; Benotti et al., 2009; Grover et al., 2011). So far, the occurrence and removal of the EDCs in STPs has been well documented around the world (Nakada et al., 2006; Heidler and Halden, 2007; Stasinakis et al., 2008). Removal could be affected by both the compound specific properties, and the factors concerning specific STPs, such as the type of treatment processes, hydraulic retention time (HRT), and the biomass particle characteristics (Carballa et al., 2004; Nakada et al., 2006; Yi and Harper., 2007; Gulkowska et al., 2008). The current data suggest that wastewater treatment processes (e.g. activated sludge) have variable performance in removing EDCs. By comparing the influent and effluent estrogen concentrations, Baronti et al. (2000) concluded that the removal efficiency for estrone (E1) was 87%. In a Brazilian STP, the observed removal efficiency for 17α-ethynylestradiol (EE2) ranged from 64% to 78% (Ternes et al., 1999). Sewage treatment processes in Italy removed 61–95% of these estrogenic compounds with median concentrations of E1 and EE2 of 9.3 and 0.45 ng L^{-1}, respectively, in effluents after treatment (Baronti et al., 2000). However, there is little data to assess the performance of STP in Tunisia in removing estrogens, which is urgently needed to minimize adverse biological effects to the aquatic life in the country. The goals of this study were to investigate the occurrence of most potent natural and synthetic estrogens (E1, E2, E3 and EE2) in the dissolved phase of two Tunisian STPs, and to determine the removal efficiencies of these compounds between alternating anoxic/aerobic and simple activated sludge processes.

II.   Experimental

2.1 Chemicals and standard solutions

All solvents used (methanol, ethyl acetate, dichloromethane and hexane) were of distilled-in glass grade (purchased from Rathburn Chemicals Ltd.,Walkerburn, Scotland). EDC standards including E1, E2, E3 and EE2, internal standard E2-d4 were obtained from Sigma and Qmx laboratories Ltd, UK, all with an isotopic purity >98%. Separate stock solutions of individual standards (1000 mg L^{-1}) were prepared in methanol, from which working standards (10 mg L^{-1}) of individual compounds and mixtures were prepared. All standards were stored at −18 ∘C. Ultrapure water was supplied by a Maxima Unit from USF Elga, UK.

2.2 Sample collection

Wastewater samples were collected from two STPs in Tunisia: Sfax and Sousse. These STPs employed similar treatment processes: a primary treatment using screening and grit settlement to remove particles coupled with a secondary biological treatment by activated sludge process. For the secondary treatment, STP at Sfax employed an alternating anoxic/aerobic process as biological treatment; while STP of Sousse employed a simple sludge process. Detailed information on the two STPs is summarized in Table 1.

To assess the occurrence and removal efficiencies of EDCs in STPs, composite samples over 24 hours from influent, aeration tank and effluent were collected in January and February 2011. To assess the daily variation in influent concentrations we performed a three day measurement campaign with the aid of automated samplers, set to collect samples at 2h intervals. All sampling was conducted in duplicate and separately taken for later extraction and gas chromatography-mass spectrometry (GC-MS) analysis. Samples were collected in pre-cleaned amber glass bottles (2 L) into which 1% formaldehyde (v/v) was added to inhibit the microbial activity, and then taken back to the laboratory for analysis. In the laboratory, the wastewater samples were acidified to pH 3.0 with 40% H_2SO_4 (v/v) and stored at 4 ℃ in refrigerator before analysis.

To monitor the purifying biomass, samples were collected from the mixed liquor at the end of the aeration tank in 50 mL plastic bottles. To ensure accurate measurement of the oxygen demand, only less than two-thirds of the volume of each bottle was occupied with the mixed liquor, and that the bottles were capped tightly.

Table 1. Operation conditions of investigated sewage treatment plants

STP	Sfax	Sousse
Rehabilitation	2005	1996
Treatment process	Activated sludge (Low load)	Activated sludge (Average load)
Treatment comments	Alternating anoxic/aerobic activated sludge	Simple activated sludge
Population equivalent	526800	300000
Average flow (m^3 d^{-1})	49500	17000
Data at simpling		
Influent flow (m^{3}d^{-1})	50000	40000
Aeration tank flow (m^3 d^{-1})	50000	8333
Effluent flow (m^3 d^{-1})	50000	17000
HRT (h)	40	4

Population equivalent IS the annual average data in 2011. HRT and the rate flow are the data at sampling

2.3 Sample analysis
2.3.1 General analysis
Total nitrogen content and COD were determined by automatic microanalysis and colorimetric method, respectively (Hachicha et al., 2009). Phosphorus content was measured calorimetrically at 430 nm using a Shimadzu U 1000 spectrophotometer. Biochemical oxygen demand (BOD5) was determined by the manometric method with a respirometer (BSB-Controlled Model OxiTop (WTW).

2.3.2 GC-MS analysis
The target estrogens in the samples, including E1, E2, E3 and EE2, were determined according to the previously method modified by Belhaj et al. (2013). Briefly, samples (1L each) were filtered using pre-combusted Whatman GF/F filter paper (0.7 μm) to remove particulate matter, and spiked with 100 ng of E2-d_2. These were ready for solid-phase extraction (SPE). First the SPE cartridges (Oasis HLB, Waters, USA) were conditioned with 5 ml of ethyl acetate to remove residual bonding agents, followed by 5 mL of methanol and ultra-pure water (2 * 5 mL). Then wastewater samples were extracted at a flow rate less than 5 mL/min, which were subsequently eluted with 10 mL of ethyl acetate solvent. These extracts were concentrated to 1–2 mL under a gentle flow of nitrogen at 45 °C. They are derived by the addition of 50 µL pyridine and 50 µL BSTFA (to produce non-polar derivatives), which were heated at 65 ± 5 °C for 30 min. These derivatives were cooled to room temperature, evaporated under a gentle stream of nitrogen to dryness and reconstituted in 100 µL of hexane ready for the analysis by GC-MS.

GC–MS analysis was performed using a gas chromatograph (Trace GC 2000, Thermoquest CE Instruments, TX, USA) coupled with an ion trap mass spectrometer (Polaris Q, Thermoquest CE Instruments, Texas, USA) and an autosampler (AS 2000, Thermoquest).

A ZB5 (5% diphenyl–95% dimethylpolysiloxane) capillary column of 30 m x 0.25 mm i.d. (0.25 μm film thickness) is used. Helium carrier gas was maintained at a constant flow rate of 1.5 mL/min. The GC column temperature was programmed from 100 °C (initial equilibrium time 1 min) to 200 °C via a ramp of 10 °C/min, 200–260 °C via a ramp of 15 °C/min, 260–300 °C via a ramp of 3 °C/min and maintained at 300 °C for 2 min. The MS operates with electron impact ionization in full-scan mode from 50 *m/z* to 600 for qualitative analysis. The inlet and MS transfer line temperatures were maintained at 280 °C, and the ion source temperature is 250 °C. Sample injection (1µL) was in splitless mode.

2.3.3 Microscopic analysis
Examinations with phase contrast microscopy were made within 3h after collection in order to avoid progressive temporal changes in density and in richness. 25 µL sub-samples of mixed liquor were taken with 100 µL automatic micropipette (Eikelboom., 2000; Zhou et al., 2006), and observed under phase-contrast illumination (100 x magnifications). The identification of species was carried out «in vivo» (Vedry., 1987).

III. Results and discussion
The dissolved concentrations of natural and synthetic estrogens and the removal rates as well as the wastewater characteristics are summarized, respectively, in Tables 2 and 3. Removal rates were calculated based on the influent and the effluent concentration. The effluent samples below the LOQ were calculated by using the value of LOQ ((E2: 0.8 ng L^{-1}; EE2: 4.0 ng L^{-1}; E3: 2.3 ng L^{-1}; Nie et al., 2009) for the effluent concentration.

3.1 Occurrence of estrogens in wastewater of Tunisian STPs
Because each of the two STPs employed distinct secondary treatment units, the concentration of estrogens processed by the two WWTPs varied. The influent concentrations of E1 were approximately similar (50 ng L^{-1}) in the two plants (Table 2). Those values are lower than those reported in Spain STPs with a median concentration of 115 ng L^{-1} (Petrovic et al., 2002) and similar to those reported in a survey conducted in Italy with a concentration of 52 ng L^{-1} (Baronti et al., 2000), but higher than those reported in surveys conducted in Paris with a concentration of 9.6 ng L^{-1} (Cargouët et al., 2004) and England with a concentration of 1.8 ng/L (Fawell et al., 2001).

Table 2. Estrogen concentrations and removal efficiencies in Tunisian STPs (mean (SD), n=2). ND = not detected.

	Sfax				Sousse			
	E1	E2	EE2	E3	E1	E2	EE2	E3
Influent	50 (1.2)	10.8 (0.3)	25 (1)	98 (2.1)	51 (0.2)	19 (0.4)	45 (0.1)	102 (1.4)
Aeration tank	53 (1)	6 (0.9)	22 (0.9)	67 (1.4)	18 (0.9)	11 (0.4)	13 (0.6)	50 (2.1)
Effluent	10 (1)	ND	ND	ND	12 (0.6)	4 (0.4)	10 (0.1)	15 (0.2)
Removal (%)	80	≥92	≥84	≥97.5	76.5	79	77.5	85.5

The influent concentrations of E2 in Sousse plant (19 ng L^{-1}) were higher than those detected in Sfax plant (10.8 ng L^{-1}) (Table 2). This might be attributed to the highest daily amounts of deconjugated E2 excreted by tourists in Sousse, thus leading to an increase in the concentrations of E3 in the Sousse plant influents. Concentrations of E2 detected in Tunisian plants were similar to the previous surveys in Japan with a range of < 0.8–21 ng L^{-1} (Komori et al., 2004) and in Italy with a range of 4.0–25 ng L^{-1} (Baronti et al., 2000).

E3, the highest concentration detected in influents of the two plants, is excreted in the largest amount by humans (Ternes and Joss, 2006). The influent concentrations of E3 in Sousse plant (102 ng L^{-1}) were slightly higher than those detected in Sfax plant (98 ng L^{-1}), which might be attributed to the highest concentrations of E2 in Sousse influents. Concentrations of E3 in Tunisian plants were generally lower than those reported by Nie et al. (2011) with a concentration of 459 ng L^{-1} and similar to those reported by Komori et al (2004) with a median value of 110 ng L^{-1}.

The influents of Sfax and Sousse plants are contaminated with synthetic estrogen EE2 (used in contraceptive pills) which are more resistant to degradation than natural estrogens. The influent concentrations of EE2 in Sousse plant (45 ng L^{-1}) were higher than those detected in Sfax plant (25 ng L^{-1}), this can be ascribed to higher consumption of the contraceptives in Sousse. These results are coherent with this reported in the literature (Miege et al., 2009).

The influent EDC concentrations changed with time during 24-h periods, as show in Fig 1.
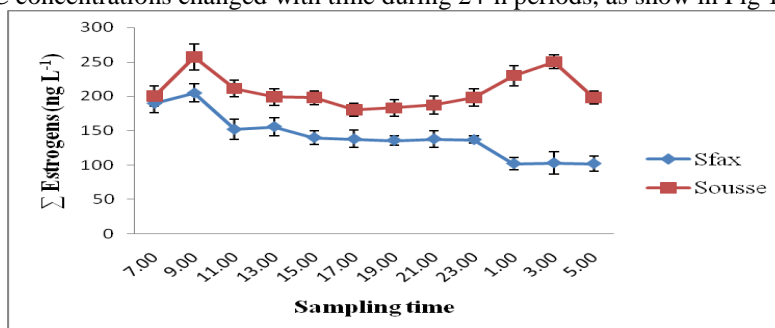
Figure 1.Comparaison of Sfax and Sousse plants influents for temporal variability of measured total steroïdal estrogens

Sfax and Sousse plants showed a total EDC concentrations (E1, E2, E3 and EE2) ranging from 190 ng L^{-1} to 205 ng L^{-1} and from 200 ng L^{-1} to 257 ng L^{-1}, respectively, between 7.00 and 11.00 am, suggesting increased discharge of estrogenic compounds in the morning and the early afternoon hours. Between 11.00 am and 23.00 pm, diurnal total EDC concentrations show a progressive decrease both in Sfax and Sousse plant ranged from 152 ng L^{-1} to 137 ng L^{-1} and from 211 ng L^{-1} to 198 ng L^{-1}, respectively.

In the midnight (23.00 pm to 5.00 am), Sfax STP shows a significant decrease of total EDC concentrations, while Sousse STP shows increment estrogens content. The reason for the significantly increase of total estrogens might be attributed to the fact that Sousse is a touristic city and very dynamic in this period. However, discharge EDCs wastewater was higher in the midnight.

The comparison between influents and effluents indicates that estrogens were not totally eliminated after the wastewater passed through the aeration tank and, as a result, were detected in most effluent samples (Table 2). In treated wastewater, E1 was detected both in Sfax and Sousse STP with a concentration of 10 ng L^{-1} and 12 ng L^{-1}, respectively. These values are similar to those reported in previous surveys in Japan with a median value of 12 ng L^{-1} (Komori et al., 2004) and in Italy with a median value of 9.2 ng/L (Baronti et al., 2000). The relatively high concentration of E1 in treated effluents can be caused by the transformation of E2 into E1 before being further transformed (Shi et al., 2004; Czajka and Londry, 2006).

E2 and EE2 were detected in effluent samples of Sousse STP with concentrations of 4 ng L^{-1} and 10 ng L^{-1}, respectively, probably due to the incomplete deconjugation of those compounds present in influent of this plant (Kirk et al., 2002). In treated wastewater of Sfax STP, Concentrations of those compounds were below the LOQ.

Regarding E3, it was detected only in the effluent from Sousse STP with a concentration of 15 ng L^{-1}, probably due to the higher concentration of E2 in the influents of this plant and the incomplete degradation in the aeration tank. This value was higher than that previously reported by Komori et al. (2004) with a median value of 1.5 ng L^{-1} and Baronti et al. (2000) with a median value of 1.4 ng L^{-1}.

3.2 Distribution of estrogens in Tunisian STPs

Fig. 2 shows the characteristic distributions of estrogens in the Tunisian STPs. In the Sousse plant, E3 had the largest portion in influents (91.25%) as well as in effluents (36.55%). As E3 is the final estrogen metabolite in human body, the Sousse-STP influent might be affected by the estrogen metabolism (Khanal et al., 2006).
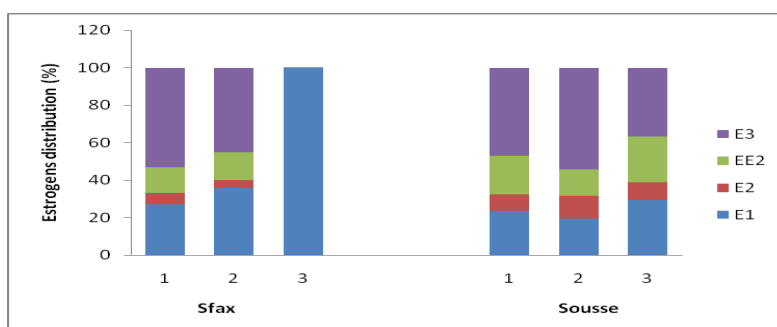
Figure 2. Distribution characterization of estrogens in wastewater from Tunisian STPs
(1: Influent; 2: Aeration tank; 3: Effluent)

The distribution tendencies of the other compounds were similar between the influents and effluents in Sousse plant. In the Sousse plant influents, the proportion of E1 (3.87%) was the second highest, followed by EE2 (3.43 %) and E2 (1.45%). Like the Sousse influents, in the Sousse effluents, E1 (29.42%) was the second highest, followed by EE2 (24.36%)) and E2 (9.66%).

The proportion of E3 (54.50%) in the aeration tank was dominantly found, which seems to be related to the highest concentration in the influents. The same distribution tendencies of influents and effluents were showed. E1 (19.66%) was the second highest, followed by EE2 (14.05%) and E2 (11.79%).

In the Sfax plant, E3 had the largest proportion in influents (62.12%) as well as in effluents (100%). However, except for E3, the distribution tendencies of other compounds were different between the influents and effluents in Sfax plant. In the Sfax plant influents, the proportion of E1 (35.5%) was the second highest, followed by EE2 (1.68%) and E2 (0.70%). The distribution of estrogens in the Sfax plant influents was different from the result in the Sousse plant influents, which might be attributed to the fact that STP of Sousse processed wastewater from various sources, including domestic, touristic and hospital waste effluents, and our findings may be reflective of the local environment.

Unlike the Sousse plant influents, only E1 was detected in the effluents of Sfax plant. Both STPs have biological treatment processes as the main treatment process, but the concentration transition of estrogens in each STP was different. In Sfax plant's aeration tank, the proportion of E3 (0.15%) was low compared with the influent wastewater which seems to be related to the biodegradation of these pollutants through the biological treatment processes (Li et al., 2005). Beside this compound, E1 (65.02%), EE2 (27.64%) and E2 (7.3%) were also found.

3.3 Daily loads of estrogens from Tunisian STPs

Fig. 3 shows the daily loads of estrogens in the STPs. Because the two plants had different number inhabitants, daily load was calculated by multiplying the total concentration of estrogens by the flow rate influents per 1000 inhabitants (Sim et al., 2011). Despite the greatest operation capacities of Sfax plant (50000 m^3 d^{-1}), Sfax and Sousse plants had approximately similar daily load of influent, (112.7 μg d^{-1}) and (109.58 μg d^{-1}), respectively. In the Sousse plant effluents, the load of estrogens (2.38 μg/d) was low compared to that in the influent, but this level was higher than the Sfax plant (0.75 μg d^{-1}). This might be attributed to the poor performance of the treatment plant at Sousse.

In Sfax plant aeration tank, the daily load of estrogens (6.15 μg d^{-1}) was higher than that in Sousse plant (1.78 μg d^{-1}), which seems to be related to the flow rate of the aeration tank. However, only the 1/3 of the average flow of Sousse plant (17000 m^3 d^{-1}) passed into the aeration tank because the plant was overloaded throughout the year.
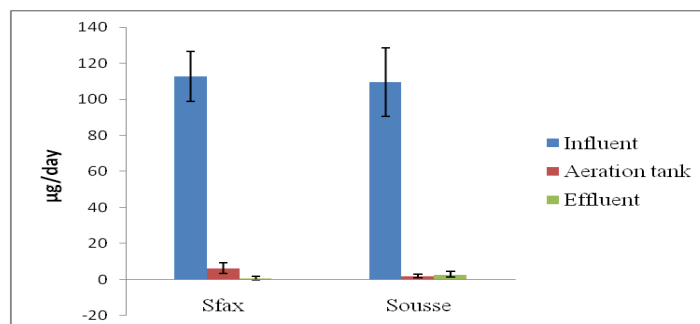
Figure 3 Daily emission amounts of estrogens in wastewater

3.4 Removal efficiencies of estrogens from the dissolved phase

      Removal of dissolved estrogens may be highly dependent on the configurations and operation conditions of individual STP as well as wastewater characteristics. The wastewater characteristics in effluent of Sfax plant show a higher removal of COD, BOD, TSS, TN and TP than the Sousse plant (Table 3), indicating a higher performance of the anoxic/aerobic process than the simple activated sludge process. As a result, an effective removal of estrogens from the Sfax wastewater was observed (Table 2).

Table 3. Wastewater characteristics (mg L^{-1}) of Tunisia STPs (n=2)

Sampling site	Sfax						Sousse				
	COD	BOD	TSS	TN	TP		COD	BOD	TSS	TN	TP
Influent	1102	499	380	70	20		775	298	260	75.9	16
	(3)	(1.8)	(1.7)	(1.2)	(0.8)		(2.1)	(1.2)	(1.3)	(1.2)	(0.9)
Effluent	100	41	30	6	1.2		114	52	45	44	6
	(0.9)	(0.7)	(1.1)	(0.8)	(0.2)		(1.3)	(0.8)	(1)	(0.8)	(0.5)
Removal (%)	90.9	91.5	92	90	94		85	82.5	82.5	42	62

      Results indicated that among the steroid estrogens, E1 showed the lowest removal in Sfax and Sousse plants, (80% and 76.5%, respectively). The removal of E1 was somewhat lower than that of the other estrogens. This could be caused by conversion of E2 to E1 in the treatment process, as other researchers have suggested (Ternes et al., 1999), or by cleavage of estrogen conjugates (Carbella et al., 2004). These results are in agreement with those reviewed by Nie et al. (2011) with a mean removal rate from the dissolved phase of around 75% and by Baronti et al. (2000) with a mean removal rate of around 61%. E3 showed the highest removal in both Sfax and Sousse plants, (97.5% and 85.5%, respectively).

      E2, E3 and EE2 can be considered as completely removed since they were not detected in the effluents. It has been reported that high HRT may contribute to an increased removal rate of estrogens (Clara et al., 2005). Sfax plant with a higher HRT (40 h), was shown to have a relatively high and stable removal of both natural and synthetic estrogens, due to increased contact time of the target compounds with microorganisms in the treatment reactors. This tendency is consistent with previous studies (Clara et al., 2005, Johnson et al., 2005, Servos et al., 2005)

      Several studies mentioned that STPs with nitrification showed a better removal of natural estrogens than STPs without nitrification (Anderson et al., 2003, Servos et al., 2005, Hashimoto et al., 2007). Sfax plant has treatment stages for the removal of nitrogen, and which makes the plant more effective for removing estrogenicity due either to the anoxic step, or more probably to prolonged duration of the biological steps.

      In the Sousse plant, all estrogens were detected in the final effluents. E1 levels were apprximately 12 ng L^{-1}, E2 was about 4 ng L^{-1}, E3 was 15 ng L^{-1}and EE2 was 10 ng L^{-1}. Despite the low concentrations measured in the plant effluents, it is important to remember that sex hormones (E1 and E2) are capable of exerting estrogenic effects at even lower concentrations than those detected in the effluents. Removal achieved for the natural estrogens was 76.5% for E1, 79% for E2 and 85.5% for E3. Short wastewaters hydraulic retention times (4 h) were considered responsible for the poor performance of the Sousse plant.

      A poor removal of synthetic estrogen was also detected in Sousse plant (77.5%). EE2 is metabolized in human body before its excretion and found then in conjugated forms (Jürgens., 2002). This conjugation increases its water solubility, and thus this compound becomes more mobile in the environment than its free form (Kozak., 2001). In addition, the presence of an ethinyl group at one hydroxyl group containing C- atom is vulnerable to microbial attack. A cleavage of this ring therefore difficult what makes EE2 much more recalcitrant in the environment. Indeed, Turan (1995) reported no change in EE2 concentration over 120 h of treatment. Moreover, Vader et al. (2000), and Yi and Harper (2007) found that under non-nitrifying conditions, there was no degradation of EE2, while nitrifying sludge oxidizing EE2 to more hydrophobic compounds. Layton et al. (2000) also observed in laboratory experiments that sludge, which failed to nitrify, also failed to

degrade EE2. The lower removal efficiencies of EE2 in Sousse-STP can be partly ascribed to their more hydrophobic nature, their highest occurrence in the influents, and their persistence under microbial attack. Also the short hydraulic retention time (4h) and the overloading of the plant could be responsible.

On the other hand, since EE2 is the more hydrophobic compound than the other estrogens, sorption to floc biomass is likely to play a significant role in removal (Johnson and Sumpter., 2001). Moreover, the floc disruption may impact the bioavailability of EE2 by making parent compound available to bacterial cells located throughout the aggregate (Yi et al., 2011). This tendency was consistent with the microscopic examination of the activated sludge flocs.

3.5 Microscopic examination of the activated sludge flocs

Microscopic examination of the activated sludge flocs taken from the aeration tanks of the STPs, as show in Fig. 4, illustrated that the flocs of Sfax STP are more compact and agglomerate than those of the Sousse STP; Sfax-STP harbored more diverse protozoa communities with higher relative abundance of crawling ciliate such us *Aspidisca* and attached ciliate such us *Vorticella*, than Sousse STP.
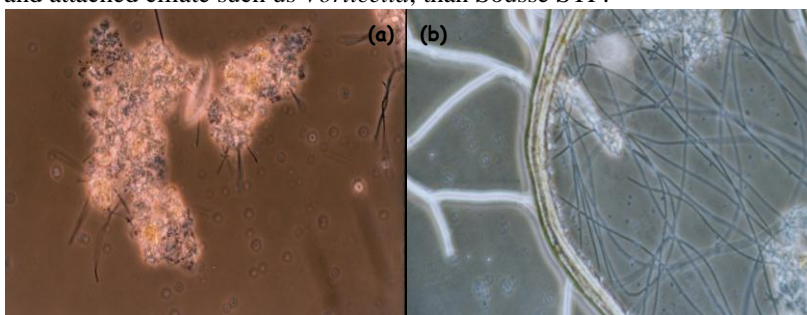
Figure 4. Microscopic examination of activated sludge flocs from Sfax-STP (a) and
Sousse-STP (b) (Magnification X 1320)

An overgrowth of filamentous microorganisms (*Nocardia* and *Thiothrix)* with right-angled branching was observed in the activated sludge of Sousse-STP, mainly due to the abundant presence of fats which can be explained by the bad functioning of fats separator, and a limited transfer of oxygen into the floc and into the purifying biomass (Martins et al., 2003). Moreover, in term of nutritional balance (C/N/P), it appears that the concentration of nitrogen at the entrance of the plant was closed to 60 mg L^{-1}, with an average BOD of 360 mg L^{-1}, there is a ratio of six times between carbon, nitrogen and phosphor. This imbalance can be regarded as among the possible causes of filamentous bacteria growth in the aeration tank of the Sousse-STP.

Nocardia had a relatively strong affinity for non-readily biodegradable fatty acids (Tsang et al., 2008). This could explain the persistence of the estrogens, especially EE2which is very hydrophobic, under the activated sludge treatment process of Sousse STP.

It is probable that removal could be affected by types of treatment process, HRT, and flocs morphology. However, nothings are known about the influence of estrogens on flocs morphology and purifying biomass in activated sludge. Further study is required to clarify this tendency.

IV.   Conclusions

The occurrence of natural and synthetic estrogens in Tunisian sewage treatment plants was investigated by sampling followed by SPE, derivatization and GC-MS analysis. The removal efficiency of estrogens in the alternating anoxic/aerobic and the simple activated sludge processes was compared. The daily loads of estrogens in the STPs using the total concentrations and flow rates of wastewater and the daily variation in influent concentrations were estimated. The microscopic examinations of the activated sludge flocs were established. The conclusions are summarized as follows:

(1) The Sousse plant, with a simple activated sludge process, showed higher concentrations of natural and synthetic estrogens in influents as well as in effluents. Removal of estrogens depend on the configurations and operation conditions of individual STP as well as wastewater characteristics. The Sfax plant with higher HRT and treatment stages for the removal of nitrogen showed a higher removal of both natural and synthetic estrogens, whereas the Sousse plant with lower HRT had a lower removal.

(2) Estrogen discharge in Sfax STP increased in the morning while Sousse STP showed increasing estrogens content in the morning and the midnight.

(3) E1 showed the lowest removal of all estrogens both in the Sfax and Sousse plants (80% and 76.5%, respectively). This was interpreted as being caused by the conversion of E2 to E1 in the treatment process, and/or by the cleavage of estrogen conjugates. The results are similar to those previously reported.

(4) E3 showed the highest removal of all estrogens both in the Sfax and Sousse plants (97.5% and 85.5%, respectively).

(5) A relatively poor EE2 removal (77.5%) was detected in the Sousse plant due to the overload of this plant, the highest concentrations of this compound in the influents, the short hydraulic retention time, and the absence of nitrifying conditions.

(6) Activated sludge microfauna was strongly affected by the operation conditions of certain system and by the presence of toxic substances in the influents such us fats which may have a stressful influence on the balance of various microorganism population, and impose growth inhibiting conditions. Filamentous bacteria were less effective in removing estrogens compared to protozoa.

Acknowledgments

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