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SUPPORTING INFORMATION

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Fate of Oestrogens during the Biological Treatment of Synthetic Wastewater in a Nitrite-Accumulating Sequencing Batch Reactor

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Use of the specific removal rate to predict oestrogen removal pathway

Responses of the specific oestrogen removal rates to different SRT of SBR1 (Figure S-1) clearly reveal the removal mechanism of natural oestrogens (E1 and E2). The pattern of specific E1 and E2 removal rates at different SRT was similar to that of COD, in which the specific removal rate was low at long SRT and increased when the SRT was reduced (Figure S-1 (a)). These results indicated that E1 and E2 were removed mainly via biodegradation by the microbial communities proliferating at SRT between 1.7 to 17.1 d. The specific EE2 removal rate at different SRT, in contrast, could not be explained by the fitted exponential curve (R^2=0.1171) if all values were considered (Figure S-1 (b)). However, when only the specific EE2 removal rates of the SRT $\geq$ 7.5 d were utilised, the obtained exponential curve was much improved (R^2=0.6682) (Figure S-1 (c)). This implied that EE2 removal mechanism could also be biodegradation and corresponded to the findings of EE2 being degraded efficiently only by specific microorganisms, growing under specific operating conditions, e.g. nitrifying bacteria at long SRT (*1, 2*).

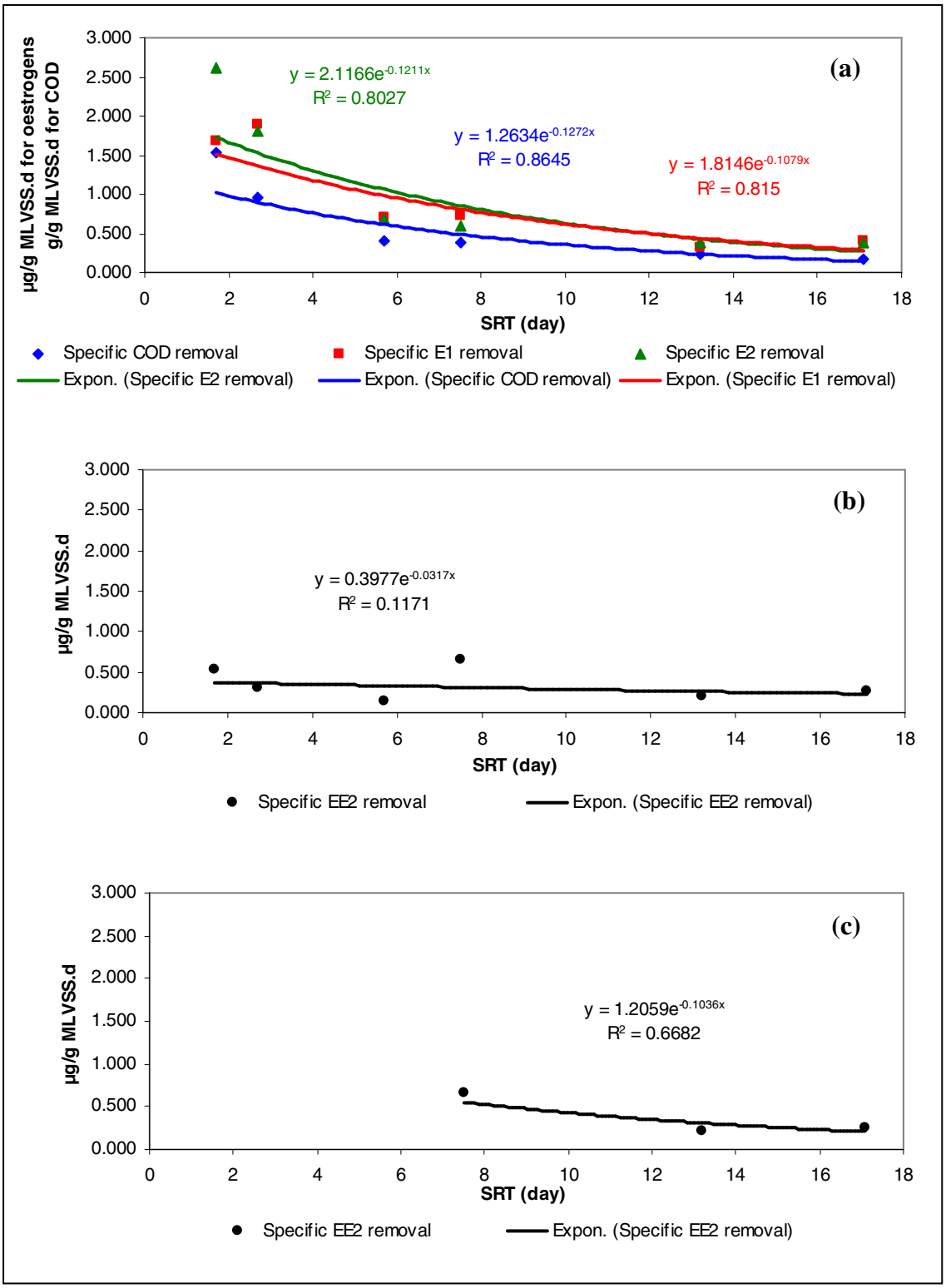

FIGURE S-1. Mean Specific COD (g/gMLVSS.d) and oestrogen (µg/gMLVSS.d) removal rate at different SRT in SBR1: for COD, E1 and E2 (a); for EE2 at all studied SRT (b); for EE2 at only when SRT ≥ 7.5 d (c)

pH conditions of SBR2

Figure S-2 shows pH valued at the end of each redox condition of SBR2. Desorption of EE2 found during the aeration phase of SBR2 was assumed to be caused by the change of pH to the level close to pK_a value which is clearly seen especially in cases of SBR2(c), where nearly 100% desorption was observed (circled in Figure S-2).

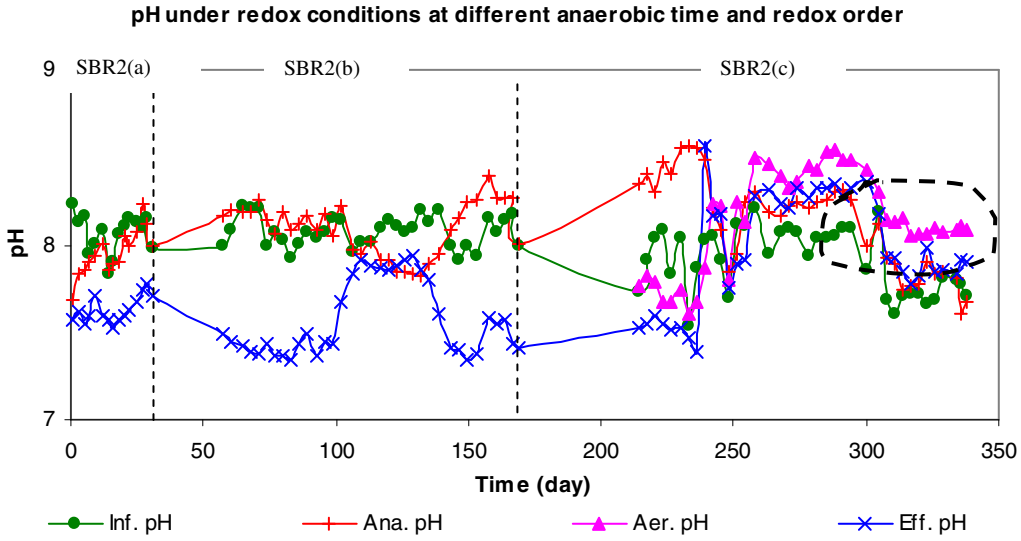

Figure S-2. pH at the end of each redox condition of SBR2 (Inf.=influent, Ana.=anaerobic, Aer.=aerobic, Eff.=effluent)

Mass flux of oestrogens in SBR1 and SBR2

Mass Flux of oestrogens used in the calculation of removal efficiencies in SBR1 and SBR2 are presented in Table S-1 and S-2, respectively.

Table S-1. Mass flux of oestrogens in SBR1

SRT (day)	Oestrogen	$F_{inf.}$[a] (ng/d)	$F_{eff.}$[b] (ng/d)	%Removal
1.7	E1	9469 (±274)	3808 (±1386)	60 (±15)
	E2	8930 (±135)	116 (±49)	99 (±1)
	EE2	9844 (±235)	8129 (±473)	17 (±3)
2.7	E1	11510 (±1659)	530 (±120)	95 (±2)
	E2	10433 (±339)	26 (±8)	100 (±0.1)
	EE2	13111 (±1054)	12026 (±2025)	9 (±8)
5.7(a)	E1	14529 (±459)	5145 (±1060)	64 (±8)
	E2	10415 (±1456)	255 (±31)	98 (±1)
	EE2	10183 (±2832)	14265 (±706)	-47 (±40)
5.7	E1	14380 (±379)	3745 (±816)	74 (±5)
	E2	10919 (±424)	240 (±100)	98 (±1)
	EE2	11485 (±815)	9835 (±673)	14 (±7)
7.5	E1	11641 (±849)	180 (±9)	98 (±0.1)
	E2	9650 (±350)	34 (±13)	100 (±0.1)
	EE2	14563 (±2312)	4172 (±165)	71 (±4)
13.2	E1	9019 (±259)	243 (±54)	97 (±1)
	E2	10341 (±302)	27 (±4)	100 (±0.04)
	EE2	9618 (±392)	4084 (±366)	57 (±6)
17.1	E1	13791 (±1054)	57 (±22)	100 (±0.1)
	E2	12650 (±909)	42 (±27)	100 (±0.2)
	EE2	14267 (±870)	5717 (±275)	60 (±4)

[a]$F_{inf.}$=influent mass flux; [b]$F_{eff.}$=effluent mass flux

Table S-2. Mass flux of oestrogens in SBR2

Experiment	Oestrogen	F_{inf}[a] (ng/d)	F_{ana}[b] (ng/d)	F_{aer}[c] (ng/d)	F_{eff}[d] (ng/d)	%Removal			
						Ana.	Aer.	Anoxic	Overall
SBR2(a)	E1	10905 (±1129)	8216 (±1219)		8448 (±706)	24 (±17)	-5 (±26)		22 (±4)
	E2	9683 (±937)	3642 (±279)		1960 (±34)	62 (±7)	46 (±4)		80 (±2)
	EE2	13821 (±2278)	8686 (±325)		8422 (±824)	36 (±10)	3 (±8)		38 (±7)
SBR2(b)	E1	12276 (±900)	3004 (±307)		220 (±71)	75 (±3)	92 (±3)		98 (±1)
	E2	11528 (±1270)	3231 (±393)		46 (±11)	72 (±5)	99 (±0.5)		100 (±0.0)
	EE2	9719 (±1553)	6920 (±792)		10220 (±1077)	28 (±11)	-48 (±2)		-7 (±17)
SBR2(c)	E1	11711 (±719)	3831 (±431)	68 (±17)	47 (±12)	67 (±5)	98 (±0.4)	32 (±4)	100 (±0.1)
	E2	10994 (±316)	1136 (±143)	22 (±12)	28 (±12)	90 (±1)	98 (±1)	-59 (±122)	100 (±0.0)
	EE2	12165 (±449)	7030 (±377)	13686 (±984)	10167 (±499)	42 (±5)	-95 (±16)	26 (±3)	16 (±6)

[a]$F_{inf.}$=influent mass flux; [b]F_{ana}=mass flux after anaerobic phase; [c]F_{aer}=mass flux after aerobic phase; [d]$F_{eff.}$=effluent mass flux

Detailed information on the analytical method for oestrogen detection

Materials and Methods

Chemicals

E1, E2, EE2 and 17α-oestradiol (the latter was used as an internal standard) were supplied from Sigma-Aldrich, UK. Acetone and hexane (GC-MS Grade) were obtained from Sigma-Aldrich, UK. Stock solutions of each oestrogen were prepared separately in acetone to a concentration of 1.0 mg/ml and stored at -20^{o}C prior to use. Anhydrous potassium carbonate (K_2CO_3) was purchased from Sigma-Aldrich, UK., and 1g of K_2CO_3 was diluted in 10 ml of deionised water. Pentafluorobenzyl bromide (PFBBr) and *N*-trimethylsilylimidazole (TMSI) were also acquired from Sigma-Aldrich, UK, and the equivalent of 0.25 g of PFBBr was diluted in 5 ml of acetone for each use. ISOLUTE 101, 200 mg/10 ml, (Argonaut IST., UK) was used for solid-phase extraction (SPE).

Solid phase extraction

OECD synthetic wastewater was prepared and an aliquot (200 ml) was filtered through a GF/A filter paper. Appropriate amounts of internal standard and oestrogens were added to the sample, and then adjusted to pH 3.5 with 1M hydrochloric acid. The same procedure was used in the preparation of effluent from a SBR. The SPE cartridge was washed with 10 ml of acetone, and then was conditioned with 10 ml of deionised water. The sample was loaded to the cartridge. The flow rate of sample through the cartridge was approximately maintained at 10 ml/min. After the extraction was completed, the cartridge was dried by applying a vacuum for approximately 30 min. The extract was eluted with acetone (3 ml), and then the eluate was evaporated to 2 ml with a gentle stream of nitrogen.

Derivatisation

One ml of the sample extract was transferred to a 10 ml glass vial. For the determination of calibration curves, 1 ml of the standard solution was used for the derivatisation. 100 µl of 10% aqueous potassium carbonate and 100 µl of 5% PFBBr reagent were added to the vial, and were kept at 60^{o}C in a water bath for 1 h. After cooling, the solvent was removed to about 100 µl with a gentle stream of nitrogen. Hexane (1 ml) was added, and the organic phase was washed with deionised water (0.5 ml). About 1 ml of the organic phase was transferred to a new 2 ml glass vial, and then the solvent was completely removed with a gentle stream of nitrogen. TMSI (50 µl) was next added to the vial. The vial was kept at room temperature for 30 min. Hexane was added up to 1 ml. Because the remaining TMSI reagent could damage the GC column, 50 µl of deionised water was added to the vial in order to remove residual traces of this reagent. The vial was then shaken until solvent in the vial became clear. About 1 ml of only hexane was transferred to a new 2 ml vial.

Instrumentation

GC-MS analysis of oestrogens was performed on a Hewlett-Packard 6890 GC split/splitless injector (260^{o}C) linked to a Hewlett-Packard 5973 MSD in CI. Methane was used as the ionisation gas at 2 ml/min in negative ion mode (electron voltage 175 eV, source temperature 240 ^{o}C, quadrupole temperature 106 ^{o}C multiplier voltage 2500 V, interface temperature 310 ^{o}C). The acquisition was controlled by a HP Chemstation software in selected ion mode for 4 ions (269, 343, 367, 431 at 1 cps 250 ms dwell) for greater sensitivity. The sample (4 µl) in hexane was pressure pulse injected by an HP7683 auto sampler and the spit opened after 1 minute. After the solvent peak had passed, the GC temperature programme and data acquisition

commenced. Separation was performed on a fused silica capillary column (15m×0.25mm i.d) coated with 0.25 μm of 5% phenyl methyl silicone (HP-5MS). The GC was temperature programmed from 150 ^{o}C-310 ^{o}C at 10 ^{o}C/min then held at final temperature for 5 min with helium as the carrier gas (flow 1ml/min, initial pressure of 35 kPa, split at 30 ml/min. The acquired data was stored on DVD for later data processing, integration and printing.

NICI-MS spectra of PFB-TMS derivatives

All pentafluorobenzyl-trimethylsilyl (PFB-TMS) derivatives of oestrogens produced an intense $[M\text{-}PFB]^-$ ion in the NICI mode (Figure S-3). These results show the advantage of NICI mode over the EI mode in terms of the capability to reduce fragmentation contributing to more sensitivity and lower detection limits (*3*) and facilitating the use of MS-MS or multiple MS detection (*4*). These were proved by the success of applying modified Nakamura et al. (*3*) derivatisation method on the quantitation of oestrogens in ground water and swine lagoons using GC-MS-MS (*5*) and in water from Australian wastewater treatment plants using GC-NICI-MS (*6*). As shown in Figure S-3, masses (m/z) of PFB-TMS derivatives used for GC-NICI-MS (SIM) are 269, 431 and 367 for E1, E3 and EE2, respectively and 343 for both E2 and 17α–oestradiol.

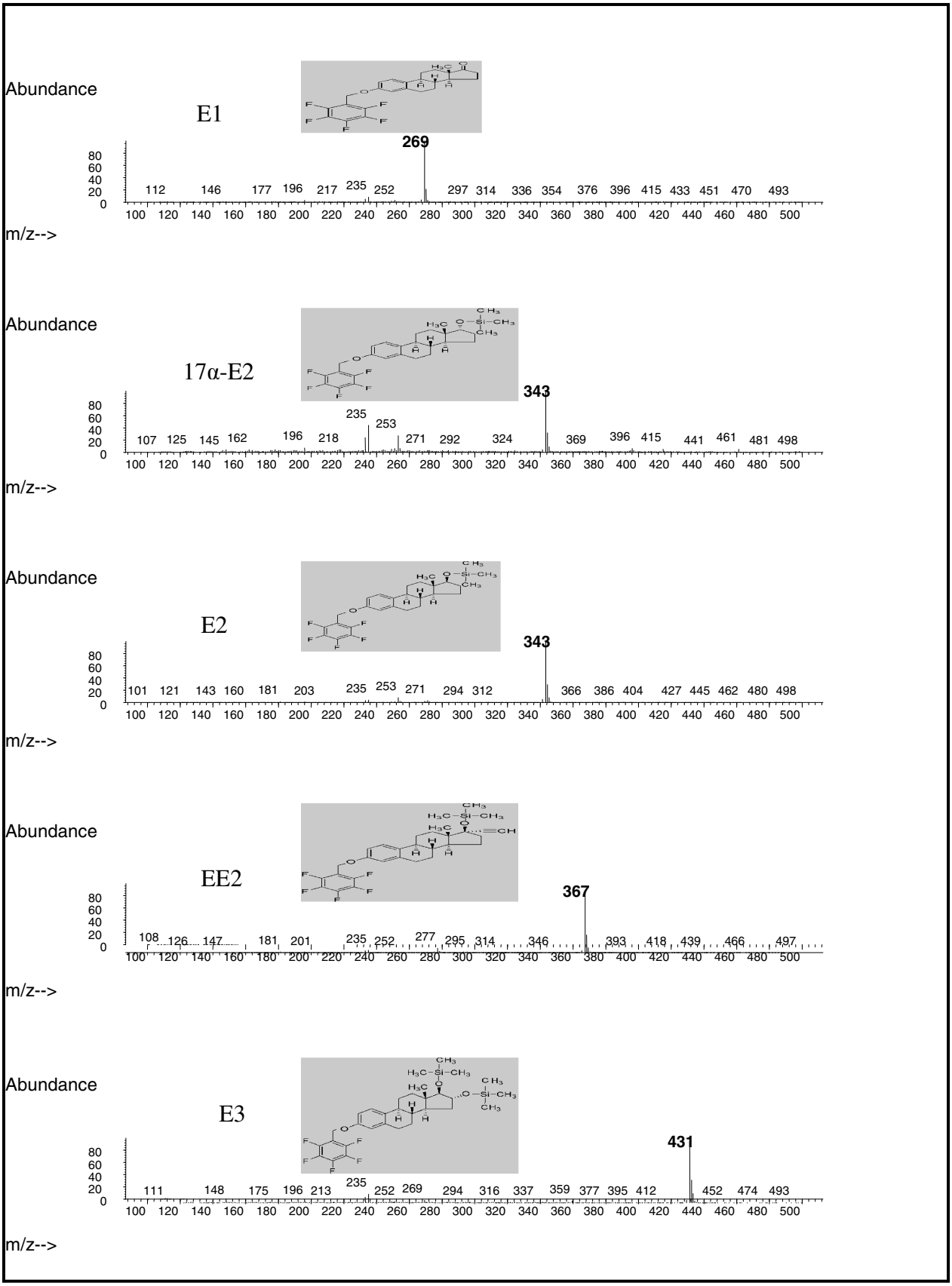

Figure S-3. NICI mass spectra of the PFB-TMS derivative

219 Reproducibility, recovery and method detection limit

220 The recovery and reproducibility of the method were determined by spiking oestrogen
221 standard solutions to attain the concentrations of 500 ng/l and 100 ng/l in 200ml of
222 OECD synthetic sewage and the effluent from a SBR. The spiked sample and a blank
223 for each matrix (prepared by adding only the internal standard in 200 ml of sample)
224 were extracted, derivatised and analysed by GC-NICI-MS, according to the procedure
225 mentioned above. The recovery and reproducibility were tested and the results are
226 listed in Table S-3. Good recovery was obtained for all oestrogens between 92.1 and
227 138.3 % for the OECD synthetic sewage sample at 500 ng/l and between 92.9 and
228 137.0 % for the SBR effluent sample at 100 ng/l. The reproducibility relative standard
229 deviation (RSD) ranged between 7.5 and 29.7% (n=5) for the OECD synthetic sewage
230 and between 4.4 and 5.8% (n=5) for the SBR effluent, showing practical and
231 acceptable reproducibility, RSD ≤ 30% (*7*).

232

233 **Table S-3.** Recovery and reproducibility (%RSD) of oestrogens from OECD
234 synthetic sewage and SBR effluent

OECD synthetic sewage *(n = 5)*	**% Recovery**	**RSD (%)**
E1	138.3	7.9
E2	92.8	7.5
E3	92.1	29.7
EE2	93.4	12.6
SBR effluent *(n = 5)*	**% Recovery**	**RSD (%)**
E1	133.9	4.4
E2	92.9	5.8
E3	137.0	4.9
EE2	94.0	5.6

235 Amounts of each oestrogen in 200 ml OECD synthetic sewage and SBR effluent were 500 and 100 ng/l,
236 respectively.
237

Method detection limit (MDL) is defined as the statistically calculated minimum amount that can be measured with 99% confidence that the reported value is greater than zero (*7, 8*) and was calculated using Equation S-1.

$$MDL = (S)(t_{(n-1,1-\alpha=0.99)}) \qquad \textbf{Equation S-1}$$

where: $t_{(n-1,1-\alpha=0.99)}$ = Student's t value for the 99% confidence level with n-1 degrees of freedom

n = number of replicates

S = standard deviation of replicate analyses

Oestrogens were added to five replicates of 200 ml of effluent SBR, used as the representative of all water samples, to a concentration of 5 ng/l. Then, the samples were extracted, derivatised and analysed. Table S-4 shows the MDLs, recovery and reproducibility of each oestrogen at the concentration of 5 ng/l.

Table S-4. Method detection limits, recovery and reproducibility (n=5) of oestrogens

Oestrogens	Concentration(ng/l)	Recovery (%RSD)	MDLs (ng/l)
E1	5.0	89.7 (11.8)	2.4
E2	5.0	87.5 (14.3)	2.9
EE2	5.0	79.2 (9.1)	1.7

Microbial diversity analysis

The analysis of microbial diversity present in the reactor of each experiment was carried out using the culture-independent method, PCR-DGGE technique. It has been suggested that, in activated sludge, a small volume of sample contains a diverse microbial community, which is, however, representative of the whole system and a single sample of an activated sludge plant should be sufficient for a plant to plant comparison (*9*). Therefore, a single sample of MLSS was collected from each experiment during the mixing step (to ensure the homogeneity of the samples) in order to study its microbial diversity. Table S-5 shows the details of samples collected from each reactor.

Table S-5. Details of samples collected from each reactor

Reactor	Sampling time	Named[b]	Studied microbial community
SBR1	Before oestrogen addition	SBR1.1	Eubacteria and AOBs
	End of SRT 7.5 d	SBR1.2	Eubacteria and AOBs
	End of SRT 13.2 d	SBR1.3	Eubacteria and AOBs
	End of SRT 17.1 d	SBR1.4	Eubacteria and AOBs
	During low DO at SRT 5.7 d	SBR1.5	Eubacteria and AOBs
	End of SRT 5.7 d	SBR1.6	Eubacteria and AOBs
	End of SRT 2.7 d	SBR1.7	Eubacteria and AOBs
	End of SRT 1.7 d	SBR1.8	Eubacteria and AOBs
SBR2	Before oestrogen addition	SBR2.1	Eubacteria and AOBs
	End of SBR2(a)	SBR2.2	Eubacteria and AOBs
	End of SBR2(b)	SBR2.3	Eubacteria and AOBs
	End of SBR2(c)	SBR2.4	Eubacteria and AOBs

[a]Names of each sample in the DGGE gel (Figure S-4 and S-5)

Bacterial and AOB community diversity of different samples was assessed using the Shannon index of general diversity (H) whilst the similarity between different samples was examined using Raup and Crick index (*10*). Comparisons among samples were made based on one DGGE analysis per one sample, since the analysis of duplicate MLVSS samples by DGGE has revealed that the profiles obtained were reproducible (*10*).

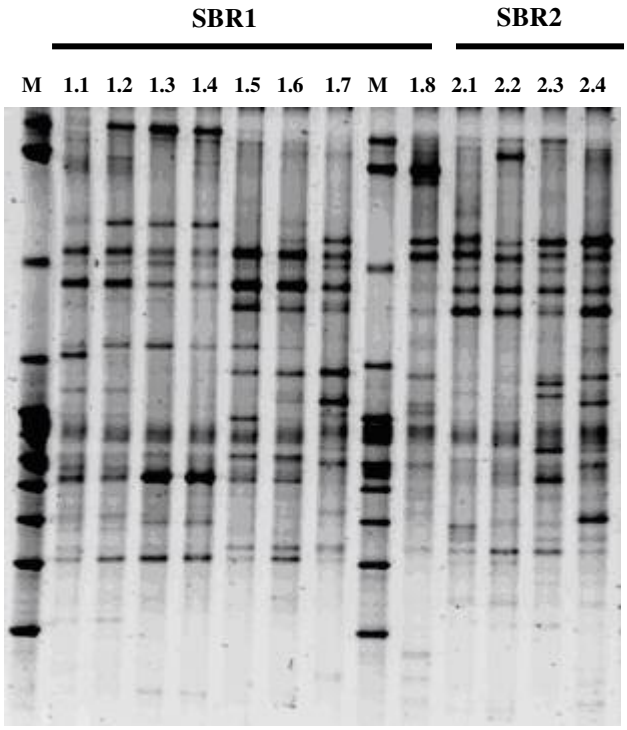

Figure S-4. DGGE profile of eubacterial communities from SBR1 and SBR2

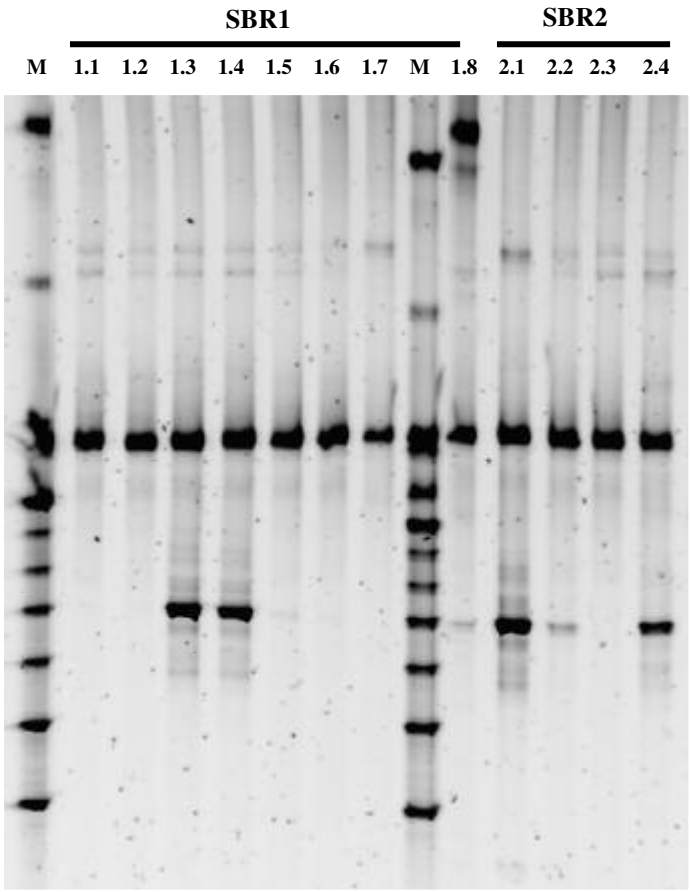

Figure S-5. DGGE profile of AOB communities from SBR1 and SBR2 (M = the marker band, run to correct for variation across the gel). Lane number definitions given in Table S-5

Shannon index of general diversity (H)

The diversity of the different bacterial and AOB communities in this study was assessed by the Shannon diversity index, which consists of two components: (i) the total numbers of species present or species richness and (ii) the distribution of the number of individuals among those different species, called species evenness or species equability (*11*). By using the diversity index, estimation of the diversity of microbial communities can be initially made, since samples with the higher H imply the greater diversity of the microbial communities (*12*). Table S-6 shows the Shannon diversity index (H) and richness (S; number of individual band) of the bacterial and AOB community from each experiment.

Table S-6. Comparison of the Shannon diversity index of the bacterial and AOB community from each experiment

Reactor	Lane	Bacterial community		AOB community	
		H	S	H	S
SBR1	SBR1.1	3.091	22	1.386	4
	SBR1.2	3.219	25	1.609	5
	SBR1.3	2.996	20	2.303	10
	SBR1.4	3.091	22	2.303	10
	SBR1.5	2.996	20	1.099	3
	SBR1.6	3.178	24	1.099	3
	SBR1.7	2.944	19	1.386	4
	SBR1.8	2.944	19	1.946	7
SBR2	SBR2.1	3.045	21	2.303	10
	SBR2.2	2.833	17	1.386	4
	SBR2.3	3.135	23	1.609	5
	SBR2.4	3.135	23	1.946	7

H indicates diversity; S indicates richness; Lane number definitions given in Table S-5

The diversity indices revealed that diversity of bacterial communities in SBR1 was not increased according to the increase of SRT. In fact, bacterial diversity of SBR1 operating at SRT 7.5 d (SBR1.2) was greater than that of the longest SRTs of this

312 reactor (13.2 and 17.1 d; SBR1.3 and SBR1.4). However, the least bacterial diversity
313 was observed in the sample at the shortest SRTs (2.7 and 1.7 d; SBR1.7 and SBR1.8).
314 In contrast to the bacterial diversity, the Shannon index values showed that the most
315 diverse AOB communities were present in SBR1 at the longest SRTs (13.2 and 17.1 d;
316 SBR1.3 and SBR1.4). Results of the microbial diversity analysis in SBR1 suggest that
317 greater diversity of the eubacteria supports a more efficient oestrogen removal
318 capability of this reactor, particularly for the recalcitrant EE2. However, EE2 removal
319 efficiency was reduced when a greater diversity of AOB was observed. Removal
320 efficiency of EE2 decreased when SRT was increased from 7.5 d to 13.2 and 17.1 d
321 because at longer SRTs greater amounts of nitrite were produced (corresponding to
322 the more diverse AOB population) which resulted in the overall bacterial diversity
323 being adversely affected (lower eubacteria diversity) probably by nitrite toxicity.
324 These effects of AOB diversity were supported by the Raup and Crick similarity
325 index, when the AOB communities of SBR1 operating at SRT 13.2 and 17.1 d were
326 statistically similar, whilst they were proved to be statistically dissimilar compared to
327 the sample taken at SRT 7.5 d, where higher EE2 removal was found (*13*). This means
328 the nitrite producing activity of AOB communities at SRT 13.2 and 17.1 d adversely
329 affected the high diversity of the eubacteria, whilst AOB community at SRT 7.5
330 produced less nitrite which did not affect the eubacteria diversity to the same extent.
331 The importance of the bacterial diversity for efficient removal of oestrogens was also
332 clearly shown when the least diverse bacterial community (at SRT 1.7 d) provided the
333 least E1 removal. Additionally, the Raup and Crick index revealed the expected
334 similarity and dissimilarity of both the eubacteria and AOB communities in the
335 experiments operated at different SRTs (*13*). For example, the AOB communities at
336 SRT 1.7 d and those at SRT 7.5, 13.2 and 17.1 d, where nitritation was observed,

were found to be significantly dissimilar and the AOB communities during the low DO conditions at SRT 5.7 d were not statistically similar to those at longer SRTs, when nitritation process was normally occurred. Therefore, changes in the oestrogen removal mechanism proposed during the experiments of SBR1 were likely to be resulted from changes to the microbial consortia over the period of each studied SRT rather than being just a difference response of the same original consortia persisting over time.

In SBR2, samples collected from SBR2(b) and SBR2(c) had the greatest bacterial diversities (SBR2.3 and SBR2.4), whilst AOB community was more diverse in SBR2 before oestrogen addition (SBR2.1) than that at the end of SBR2(a) experiment (SBR2.2). Moreover, diversity of AOB community in SBR2(c) (SBR2.4) was also found to be greater than that in SBR2(b) (SBR2.3). The results imply that microbial diversity also affected the performance of SBR2 in oestrogen removal. Conditions with greater microbial diversity (SBR2(b) and SBR2(c)) provided better E1 and E2 removal efficiencies.

Literature Cited in Supporting Information

(1) Shi, J.; Fujisawa, S.; Nakai, S.; Hosomi, M., Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium Nitrosomonas europaea. *Water Research* **2004,** 38 (9), 2323-2330.

(2) Vader, J. S.; van Ginkel, C. G.; Sperling, F. M. G. M.; de Jong, J.; de Boer, W.; de Graaf, J. S.; van der Most, M.; Stokman, P. G. W., Degradation of ethinyl estradiol by nitrifying activated sludge. *Chemosphere* **2000,** 41 (8), 1239-1243.

(3) Nakamura, S.; Hwee Sian, T.; Daishima, S., Determination of estrogens in river water by gas chromatography-negative-ion chemical-ionization mass spectrometry. *Journal of Chromatography A* **2001,** 919 (2), 275-282.

(4) Lerch, O.; Zinn, P., Derivatisation and gas chromatography-chemical ionisation mass spectrometry of selected synthetic and natural endocrine disruptive chemicals. *Journal of Chromatography A* **2003,** 991 (1), 77-97.

(5) Fine, D. D.; Breidenbach, G. P.; Price, T. L.; Hutchins, S. R., Quantitation of estrogens in ground water and swine lagoon samples using solid-phase extraction, pentafluorobenzyl/trimethylsilyl derivatizations and gas chromatography-negative ion chemical ionization tandem mass spectrometry. *Journal of Chromatography A* **2003,** 1017 (1-2), 167-185.

391 (6) Braga, O.; Smythe, G. A.; Schäfer, A. I.; Feitz, A. J., Fate of steroid estrogens in
392 Australian inland and coastal wastewater treatment plants. *Environmental Science and*
393 *Technology* **2005,** 39 (9), 3351-3358.
394
395 (7) Munch, J. W., *Determination of Organic Compounds in Drinking Water by*
396 *Liquid-Solid Extraction and Capillary Column Gas Chromatography/Mass*
397 *Spectrometry (U.S. EPA METHOD 525.2)*. National Exposure Research Laboratory,
398 Office of Research and Development, U.S. Environmental Protection Agency:
399 Cincinnati, Ohio 45268, 1995.
400
401 (8) Berthouex, P. M.; Brown, L. C., *Statistics for Environmental Engineers*. Lewis
402 Publishers, New York: 1994.
403
404 (9) Curtis, T. P.; Craine, N. G., The comparison of the diversity of activated sludge
405 plants. *Water Science and Technology* **1998,** 37 (4-5), 71-78.
406
407 (10) Rowan, A. K.; Head, I. M.; Snape, J. R.; Fearnside, D.; Barer, M. R.; Curtis, T.
408 P., Composition and diversity of ammonia-oxidising bacterial communities in
409 wastewater treatment reactors of different design treating identical wastewater. *FEMS*
410 *Microbiology Ecology* **2003,** 43 (2), 195-206.
411 (11) Kennedy, A. C.; Smith, K. L., Soil microbial diversity and the sustainability of
412 agricultural soils. *Plant & Soil* **1995,** 170 (1), 75-86.
413 (12) Boon, N.; De Windt, W.; Verstraete, W.; Top, E. M., Evaluation of nested PCR-
414 DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA

415 primers for the analysis of bacterial communities from different wastewater treatment
416 plants. *FEMS Microbiology Ecology* **2002,** 39 (2), 101-112.
417
418 (13) Pholchan, P.; Sallis, P. J., Relationships of microbial communities and oestrogen
419 removal during wastewater treatment in the nitrite accumulating sequencing batch
420 reactor (in preparation).
421
422
423