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Influence of Hydraulic Retention Time, Sludge Retention Time and Ozonation on the Removal of Free and Conjugated Estrogens in Japanese Activated Sludge Treatment Plants

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Abstract

This study describes the occurrence, fate and removal of free estrogens [estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethynylestradiol (EE2)] and their glucuronide and sulphate conjugates [estrone-3-sulphate (E1-3S), 17β-estradiol-3-sulphate (E2-3S), estriol-3-sulphate (E3-3S), estrone-3-glucuronide (E1-3G), 17β-estradiol-3-glucuronide (E2-3G) and estriol-3-glucuronide (E3-3G)] in twelve sewage treatment plants (STPs) in Japan. Glucuronide conjugates were only rarely detected in sewage influent and entirely eliminated within the treatment plants. E1 was found at 69 ng/L, E2 at 108 ng/L, E1-3S at 18 ng/L and E2-3S at 78 ng/L in sewage influent. Average removal efficiency for E1, E2 and sulphate conjugates was 88, 92 and 93%, respectively following activated sludge treatment. The removal of E1 and E2 was improved with increasing sludge retention time (SRT), with the highest removal typically found from 12 days SRT onwards. The removal of sulphate conjugates was also related to SRT with highest removals found from 8 days SRT onwards. No correlation was found between hydraulic retention time (HRT) and the removal of any of the estrogens. Ozonation (4-7 mg/L) reduced E3 and E2-3S and E3-3S to below detection levels. Overall ozonation reduced the estrogenicity of the effluent as expressed as estradiol equivalents from 8.4 ng/L to 0.7 ng/L. The results suggest adequate river basin management of estrogens in Japan could be accomplished by a mixture of activated sludge plants with long SRT and where necessary the addition of tertiary ozonation.

Keywords:

Free estrogens; Conjugated estrogens; Activated sludge treatment; Sludge retention time; Hydraulic retention time; Ozonation

1 INTRODUCTION

The disruptive impact of steroid estrogens on wild fish and the role played by sewage treatment plants in their discharge is well known [1]. These natural free estrogens have been found to have endocrine disruptive effects on fish even at low ng/L levels [2]. The major source is believed to be the human excretion of free estrogens [2,3]. However, the majority of these estrogens are excreted as conjugates with very limited biological activity [4].

Steroid estrogens in the free de-conjugated state have been detected in sewage treatment plants (STP) discharge waters worldwide [5–11], implying that de-conjugation occurs prior to and/or during wastewater treatment. The conjugated estrogen, depending on the type of attached ester group (glucuronide or sulphate), can potentially de-conjugate back to the active free estrogens [12,13]. For example, elevated levels of estrone (E1) are suspected to arise from transformation of 17β-estradiol-3-sulphate (E2-3S) into E1 and 17β-estradiol (E2) in the activated sludge [13]. However, the de-conjugation of glucuronated and sulphated conjugates back into their free estrogenic forms occurs at different rates [12,13]. Thus, the de-conjugation process plays a key role in the overall estrogenic potency of sewage effluents and rivers. There are some studies on the fate of conjugates within sewage treatment plants [7,8], but little is known about the influence of different treatment process parameters on their removal.

Removal of estrogens in sewage treatment is largely a biological process [14,15] and so differences in this part of treatment will have a large effect on the outcome. Such factors include, temperature, dissolved oxygen concentration, substrate loading rate

[16] hydraulic retention time (HRT) and sludge retention time (SRT) [17,18]. A minimum sludge retention time of 10 to 12.5 days has been suggested as the period required for the growth of micro-organisms that decompose E2 and E1 [19]. It is not clear; however, to what extent the conditions which favour the removal of free estrogens influence also the fate of the conjugated estrogens. The two groups of compounds have different physico-chemical characteristics with the free estrogens having relatively high octanol-water partition coefficients of 3-4 Log Kow [5], whilst the conjugated estrogens are highly polar [6,7].

In Japan, particularly in Kinki area (Shiga, Kyoto and Osaka), sewage systems have mostly employed conventional activated sludge process (CAS) in middle and large scale STPs [20]. The CAS is the most widely used process for the treatment of sewage because of its low operation cost and high performance in Japan. However, the operational parameters (SRT, HRT and Dissolved oxygen) and the treatment steps can differ from one plant to another. To the best of our knowledge, this is the first study to report detail exercise on removal of conjugated estrogens in activated sludge as well as in advanced wastewater treatment process. Twelve activated sludge treatment processes in Kinki area (Japan) were surveyed to investigate the following:

1. The removal of dissolved natural free and conjugated estrogens within the twelve Japanese STPs.
2. The effect of HRT and SRT on the removal performance of STPs on free and conjugated estrogens.
3. Effect of ozonation on free and conjugated estrogen reduction.

2 MATERIALS AND METHODS

2.1 Survey in Japanese STPs

Twelve activated sludge treatment systems in Japan (Shiga, Kyoto and Osaka) each with slightly different treatment conditions were investigated. A total of 77 wastewater samples (influent=28, secondary effluent=28, final effluent=21) were collected from these STPs. The descriptions of each STP and their operational parameters are given in Table 1. It should be noted that the HRT reported here is the biological step contact time only, not that of the whole process. Out of these twelve plants, three STPs use ozonation as a tertiary treatment process followed the conventional and/or advanced activated sludge process. The ozonation contact time were between 12 to 23 min. Wastewater samples were collected from each STP in three sampling campaigns (November, 2007, November, 2008, and September, 2009). All the plants were sampled in dry weather conditions. The sampling took place at the influent, secondary effluent and effluent stages of the plants concurrently. Population equivalents ranged from 33,900 to 775,500 and the flow rate from 9,500 to 576,265 m^3/d. The SRT ranges from 3.8 to 22 days with an average of 13 days, whilst the average HRT was 8.7 h (2.4 to 14.1 h).

Twenty-four hour composite samples were collected using an automatic flow proportional sampler at 4°C. From the sampler, samples were collected in 1 L pre-cleaned amber glass bottles and immediately 1 gram ascorbic acid was added to prevent further biodegradation. All samples were transported to the laboratory in a cooler box maintained at 4°C. The filtration and concentration process of the samples were completed within 24 h of sample collection. Sludge samples were not analyzed in this study.

2.2 Sample pre-treatment and chemical analysis

The free and conjugated estrogens; E1, E2, estriol (E3), 17α-ethynylestradiol (EE2), estrone-3-sulphate (E1-3S), E2-3S, estriol-3-sulphate (E3-3S), estrone-3-glucuronide (E1-3G), 17β-estradiol-3-glucuronide (E2-3G) and estriol-3-glucuronide (E3-3G) were all obtained from Sigma-Aldrich, Japan. The deuterated standards for each compound were obtained from CDN Isotopes. All the chemicals were analyzed by the ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC/MS/MS) [21]. The sample pre-treatment development has been described in some detail previously [21]. Briefly, samples were first filtered with GF/B glass fibre filter (1 μm pore size; Whatman, UK). After adding the appropriate amount of all the deuterated surrogates, solid phase extraction (SPE) with Oasis HLB (200 mg, 6 cc; 30 μm partial size, Waters, UK) cartridges was performed. The SPE cartridges were dried for 1 h under the gentle air pressure in a glass manifold. A Sep-Pak Plus NH2 (360 mg, aminopropyl, 55–105 μm partial size, waters) cartridge was connected below the dried Oasis HLB cartridge to reduce the effect of sample matrix on ionization.

Free estrogens were eluted first from the cartridge using methanol, followed by 0.5% NH_4OH methanol to elute the conjugated estrogens [21]. Chromatographic separations were carried out on a Waters Acquity UPLC system (Milford, MA) using an Acquity BEH C8 column (100 mm, 2.1 mm, 1.7 μm particle size) for free and Acquity BEH C18 column (50 mm, 2.1 mm, 1.7 μm particle size) for conjugated estrogens. Separation was performed with a binary mobile phase (acetonitrile: milli Q) at a flow rate of 0.2 mL/min [21]. UPLC/MS/MS with electrospray ionization in the negative ionization mode was used in this study. The method parameters are

147 shown in the Supporting Information (Table S1). The detection limits of studied
148 estrogens were between 0.2 (sulphate conjugates) and 0.8 ng/L (E3-3G). Recovery
149 rates of each deuterated surrogates were between 65 (E2 in influent) and 108% (E1-3S
150 in influent) (Table S2).
151
152 **(Insert Table 1)**
153
154 **2.3 Removal calculations**
155 Percentage removal of estrogens during sewage treatment is used as a collective term
156 to describe the disappearance of chemicals from the effluent due to processes such as
157 biodegradation and sorption on sludge. The degree of removal obtained was
158 calculated from the total analyte concentration in raw sewage water (Cin) and effluent
159 (Cout) according to Eq. 1:
160 $$\text{Removal}(\%) = \frac{(\text{Cin} - \text{Cout})}{\text{Cin}} \times 100 \quad (1)$$
161 Statistical analysis was performed by using commercially available statistical software,
162 Statistica (Statsoft, Tulsa, OK, USA).
163
164 **2.4 Estradiol equivalents (E2 equiv) calculations**
165 To address the estrogenic potency for a mixture of natural estrogens (E1 and E2) in
166 terms of E2 equiv was calculated as follows [22].
167 $$\text{E2 equiv} = [\text{E2}] + [\text{EE2}] \times 10 + [\text{E1}]/3 \quad (2)$$
168 These E2 equiv values were used for the investigation of the impact of SRT and
169 ozonation in the removal of estrogenic activity within the STPs.
170
171

3 RESULTS AND DISCUSSION

3.1 Occurrence of free and conjugated estrogens in the wastewater samples

Among the free estrogens, E2 was detected in the range of 5 to 108 ng/L (36 ng/L mean) in influent samples but was detected only in few secondary effluents (up to 9 ng/L) samples. E1 was detected in the range of 11 to 69 ng/L (30 ng/L mean) in influent, and 1 to 36 ng/L (3 ng/L mean) in final effluent samples (data not shown). High effluent concentration may reflect de-conjugation of conjugated metabolites during the treatment process[13]. Reported E1 and E2 concentrations are in accordance with previous observations [21,23,24]. E1 values were similar to the previously reported concentrations in Japanese STP, where a range of 66 ng/L (Influent) and 80 ng/L (effluent) was reported. Synthetic estrogen EE2 was never been detected in any sample. This is not unexpected as it is still not a popular method of contraception in Japan [20]. E3 was detected only in influent sample (64 ng/L mean), implying a ready biodegradability during the sewage treatment process.

(Insert Figure 1)

The glucuronide conjugates were found in only two influent samples where E1-3G was found at 3.7 ng/L and E2-3G at 3.5 ng/L concentration. In a previous study, E1-3G was detected at 5 ng/L, E2-3G at 4.0 ng/L and E3-3G at 19.0 ng/L in three UK STPs influents [8]. However, D'Ascenzo et al, [25] failed to detect E3-3G, but reported E2-3G at 5.0 ng/L and E1-3G at 4.0 ng/L (mean concentration) in six Italian activated sludge plant influents. Sulphate conjugates were frequently observed in these Japanese STPs influents and secondary effluents (Figure 1) with E2-3S reaching up to 78.0 ng/L (mean concentration 19.4 ng/L) in the influent. E1-3S was detected in

197 influent and few secondary effluent samples at a mean concentration of 8 ng/L (0.8 to
198 18 ng/L) and 3.4 ng/L (<0.2 to 4.4 ng/L), respectively. This is similar to a previous
199 survey in an STP in Japan where a range of 7.7 ng/L (E1-3S) and 36.1 ng/L (E3-3S)
200 in the influent were reported [7]. In an another study in Japan, E1-3S was detected
201 upto 2.2 ng/L in STP effluent and 0.3 to 0.9 in different river and lake water samples
202 [26]. E3-3S was never been detected in any effluent samples, however it was detected
203 upto 19 ng/L in influent samples.
204
205 **3.2 Effect of SRT on estrogen and conjugates removal**
206 The E1, E2 and E2 equiv (Eq. 1) removal efficiencies were assessed and compared for
207 each of the STPs in the survey. Results denoted that the longer SRT achieved highest
208 estrogen removal and E1 took longest time to be eliminated completely. Concurrently,
209 there did appear to be a significant relationship (p<0.05) between SRT and the
210 removal of free estrogens (Figure 2). The highest removal rates could be found from
211 12 d SRT onwards. Mean removal rates of more than 84 and 98% were observed for
212 E1 and E2, respectively, at SRT higher than 12 days. In addition, consistent removal
213 for studied estrogens was observed over 18 days SRT. Previously, more than 90%
214 removal was observed for natural estrogens at SRT of 12-15 days, however in the
215 membrane bioreactors with nitrification and denitrification processes [15]. Whilst,
216 mean removal was 65% for E1 and 85% for E2, at SRT lower than 12 days. This is
217 not to say that SRT periods greater than 12 d guaranteed high removal, but that the
218 highest removal rates were most likely to occur in plants beyond this sludge age.
219 There also seemed to be a significant relationship (p<0.05) between SRT and removal
220 of the sulphate conjugates, E1-3S (r^2=0.36) and E2-3S (r^2=0.42). However, in this

221 case the highest removals appeared to be from 8-9 d SRT onwards (more than 99%
222 removal).

223 **(Insert Figure 2)**

224 A high sludge age will provide a greater opportunity for slow growing
225 microorganisms to establish themselves on the sludge flocs and these have been
226 linked to estrogen biodegradation [15,27]. It seems that species capable of degrading
227 E2 are relatively common in activated sludge [28,29] but this is not the case for E1.
228 However, there is evidence that specialist nitrifying bacteria can degrade E1 and these
229 are favoured by long sludge ages [30,31]. These results imply that increasing sludge
230 age with their attendant bacteria also encourages sulphate conjugate degradation.
231 Recent microcosm studies suggest that a significant proportion of E2-3S can be
232 transformed to the free E1 hormone [13].

233

234 **3.3 Effect of HRT on estrogen and conjugates removal**

235 The HRT in the 12 STPs varied from 7 h to 14 h. No relationship between HRT on the
236 removal of the estrogens and their sulphate conjugates was visible (Figure 3). Positive
237 correlation with SRT and not with HRT could be explained by sorption of free
238 estrogens on the sludge. In terms of polarity, free estrogens [log Kow between 2.45
239 (E3) to 4.01(EE2)] would be considered as moderately hydrophobic compounds [5]
240 and the higher proportion of sorption to sewage particles in wastewater might be
241 expected. However, in case of more hydrophilic sulphate conjugates, bacteria and
242 enzymes can hydrolyze these to yield the free estrogens particularly in activated
243 sludge process [13]. Similar results were reported by Gomes et al, [12], however,
244 using a slightly different media (artificial activated sludge) in a microcosm study.

245 **(Insert Figure 3)**

3.4 Ozonation and ecotoxicological risk assessment

The STPs; B, C and G having ozonation as a tertiary treatment process were examined in 6 sampling campaigns (Table 2). For these STPs the average E2 equiv removal efficiencies from the influent samples was almost 96% (Table 2). As far as ecotoxicological risk is concern, ozonation reduced the estrogenicity of the effluent as expressed in E2 equiv from 8.4 ng/L to 0.7 ng/L. This indicates that ozonation could reduce the E2 equiv below the threshold (1 ng/L) to cause endocrine disruption to aquatic organisms [22], as 1 ng/L E2 equiv level in the UK is taken as a trigger level for vitellogenin (VTG) production in male fish. Except on one occasion (E1-3S, 0.5 ng/L), sulphate conjugates were never detected in effluent samples having ozonation as a tertiary treatment. Previously, ozonation has been found extremely efficient at removing estrogens from the wastewater treatment plants [32,33].

(Insert Table 2)

4 CONCLUSIONS

Occurrence and removal of the estrogens and their conjugates were investigated in twelve STPs in Japan. This study demonstrated that sulphate conjugates are readily degraded in Japanese STPs with their removal promoted by longer SRT periods. The HRT of an activated sludge plant had no bearing on its estrogen removal efficiency. On a practical level, this survey suggests that where estrogen removal performance is under consideration, then an SRT above 12 d is desirable, with above 18 d being particularly effective. Ozonation has proved to be particularly effective in estrogen removal whilst conventional activated sludge plants being sufficiently effective with longer SRTs.

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391 **Figure Captions**
392
393 Figure 1 Concentration (mean) of free and conjugated estrogens in 12 activated sludge
394 treatment plants in Japan (n=28); error bar shows range of the detection.
395
396 Figure 2 Correlation plot of SRT against the E1, E2, E2 equiv, E1-3S and E2-3S
397 removal for 12 Japanese STPs (n=28).
398
399 Figure 3 Correlation plot of HRT against the E1, E2, E2 equiv, E1-3S and E2-3S
400 removal for 12 Japanese STPs (n=28).

Table 1 Detailed description of the surveyed STPs in Japan (Kyoto, Shiga and Osaka)

ID		Treatment	Physicochemical	HRT (h)	SRT (Day)	Sampling Time	Flow (m3/d)	Served population
A	A-1	CAS with coagulation	Chlorination	5.6	18.4	2007/11	57,000	99,000
	A-2	AO with carrier		2.8	14.2	2008/12		
B	B-1	Step AO	Ozonation(4.3mg/L)	9.9	22	2007/11	65,210	84,000
	B-2	Pure O2		4.6	9.5	2008/11		
C		CAS; AO	Ozonation(7.4 mg/L)	9.8	8.3	2007/11	92,280	146,500
						2008/11		
D	D-1	CAS;AO;A2O;stepAO	Chlorination	12.1	19	2007/11	576,265	775,500
	D-2			11.6	16	2008/11		
	D-3			9.4	18	2009/9		
E		AO with coagulation stepAO with coagulation	Chlorination	10.9	17	2007/12	50,000	236,000
						2008/12		
F		AO with coagulation stepAO with coagulation	Chlorination	14.1	13.1	2007/12	9,500	33,900
						2008/12		
G		AO with coagulation A2O with coagulation stepAO with coagulation	Ozonation	9.4	12	2007/12	200,000	604,000
						2008/12		
H		AO with coagulation stepAO with coagulation	Chlorination	9.9	13	2007/12	39,000	110,000
						2008/12		
I		CAS	Chlorination	7.0	16	2008/11	87,140	210,400
J	J-1	CAS(quasi-AO)	Chlorination(1.4mg/L)	6.5	6.7	2009/9	191,276	263,137
	J-2	CAS	Chlorination(2.0mg/L)	7.0	3.8			
K		AO	Chlorination(2.7mg/L)	8.6	5.0	2009/9	160,544	158,018
L		AO	Chlorination(1.4mg/L)	8.4	6.3	2009/9	135,833	210,108

CAS= conventional activated sludge
AO = anoxic/oxic
A2O = anaerobic/anoxic/oxic
Step AO = anoxic/oxic/anoxic/oxic

Table 2 Estradiol equivalent (E2 equiv) and sulphate conjugates concentrations (ng/L) in sewage treatment plants samples having ozonation process as a tertiary treatment.

	Sampling event	STP B1			STP C			STP G		
		Inf.	**S. Eff.**	**Eff.**	**Inf.**	**S. Eff.**	**Eff.**	**Inf.**	**S. Eff.**	**Eff.**
E2 Equiv	1st	18.4	6.2	1.7	37.5	9.1	0.7	122.1	10.8	1.2
	2nd	14.1	10.6	ND	31.3	7.4	ND	93.1	6.1	0.4
E1-3S	1st	3.2	ND	ND	4.7	ND	ND	11.2	3.2	0.5
	2nd	1.6	ND	ND	5.2	ND	ND	7.8	ND	ND
E2-3S	1st	1.8	ND	ND	9.2	ND	ND	61.5	0.8	ND
	2nd	2.3	ND	ND	8.3	ND	ND	41.0	0.4	ND
E3-3S	1st	6.2	ND	ND	17.9	ND	ND	10.9	0.3	ND
	2nd	8.2	ND	ND	18.7	ND	ND	9.2	0.3	ND

ND= Not Detected; Inf.= Influent; S. Eff.= Secondary Effluent; Eff.= Effluent

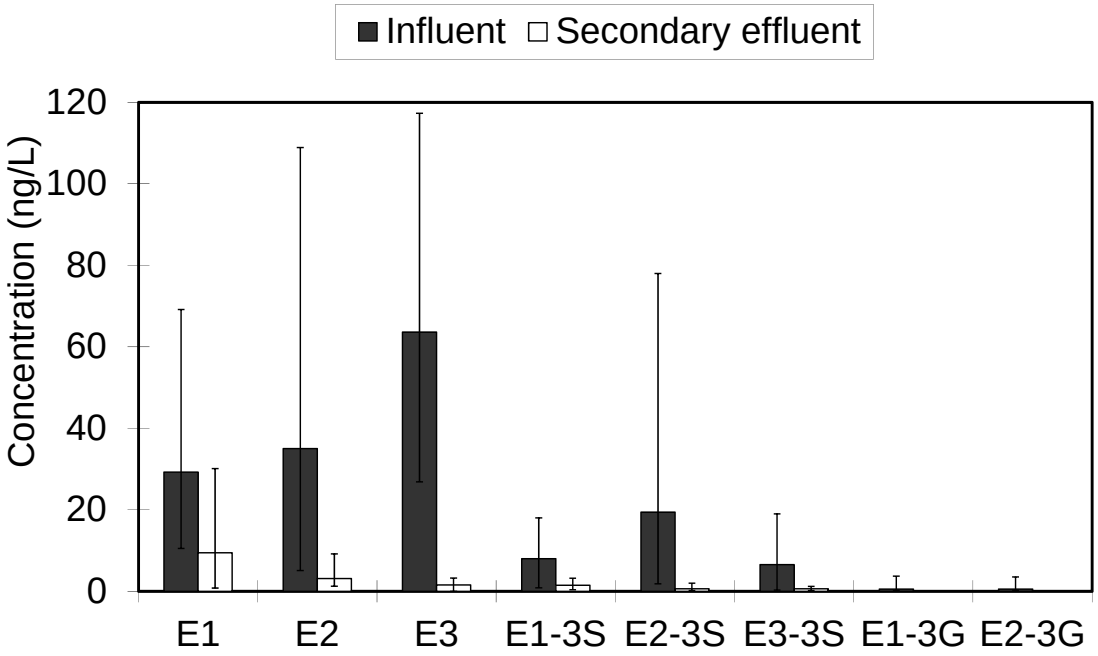

Figure 1 Mean concentration of free and conjugated estrogens in 12 activated sludge treatment plants in Japan (n=28); error bar shows range of the detection.

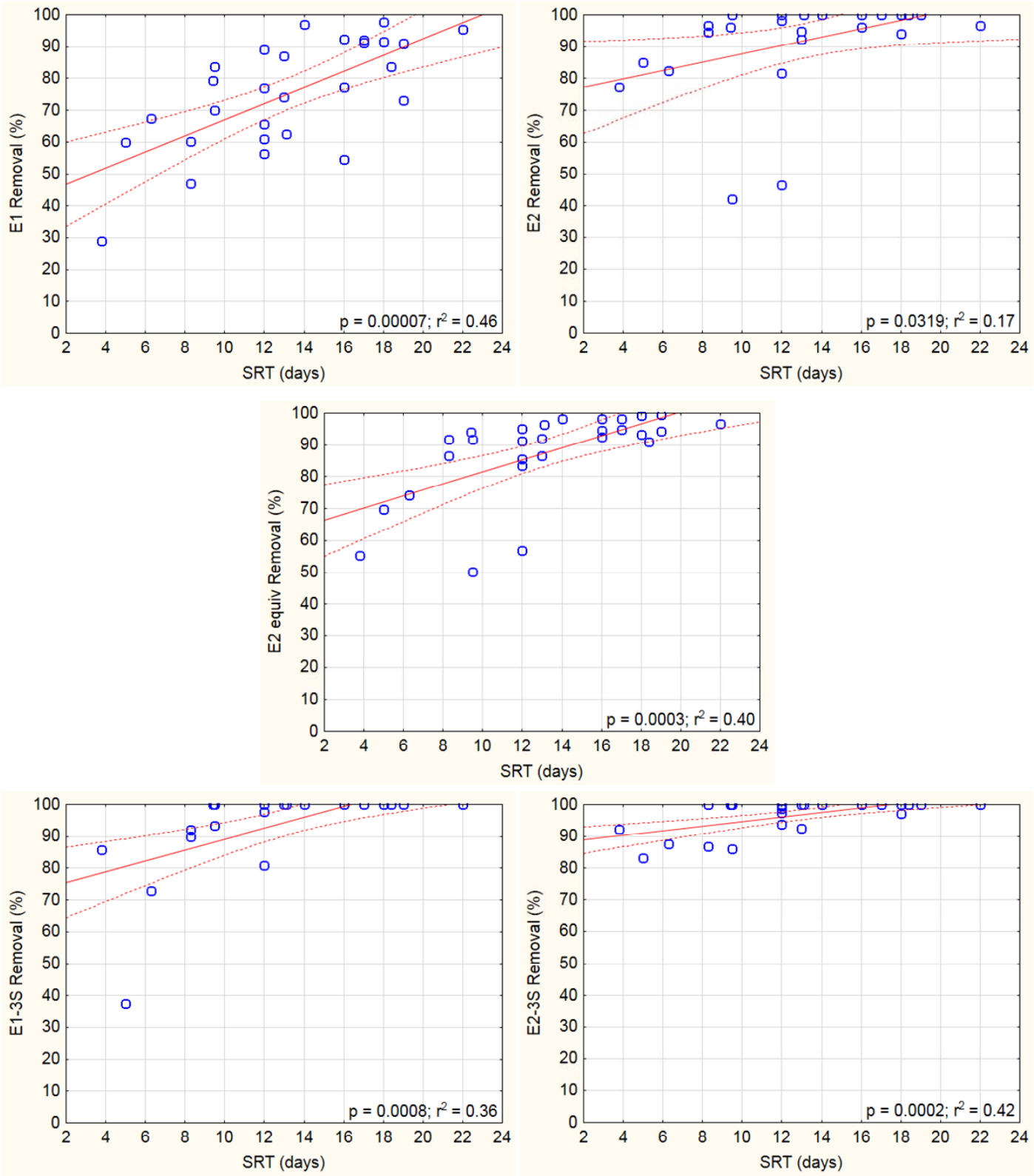

Figure 2 Correlation plot of SRT against the E1, E2, E2 equiv, E1-3S and E2-3S removal for 12 Japanese STPs (n=28).

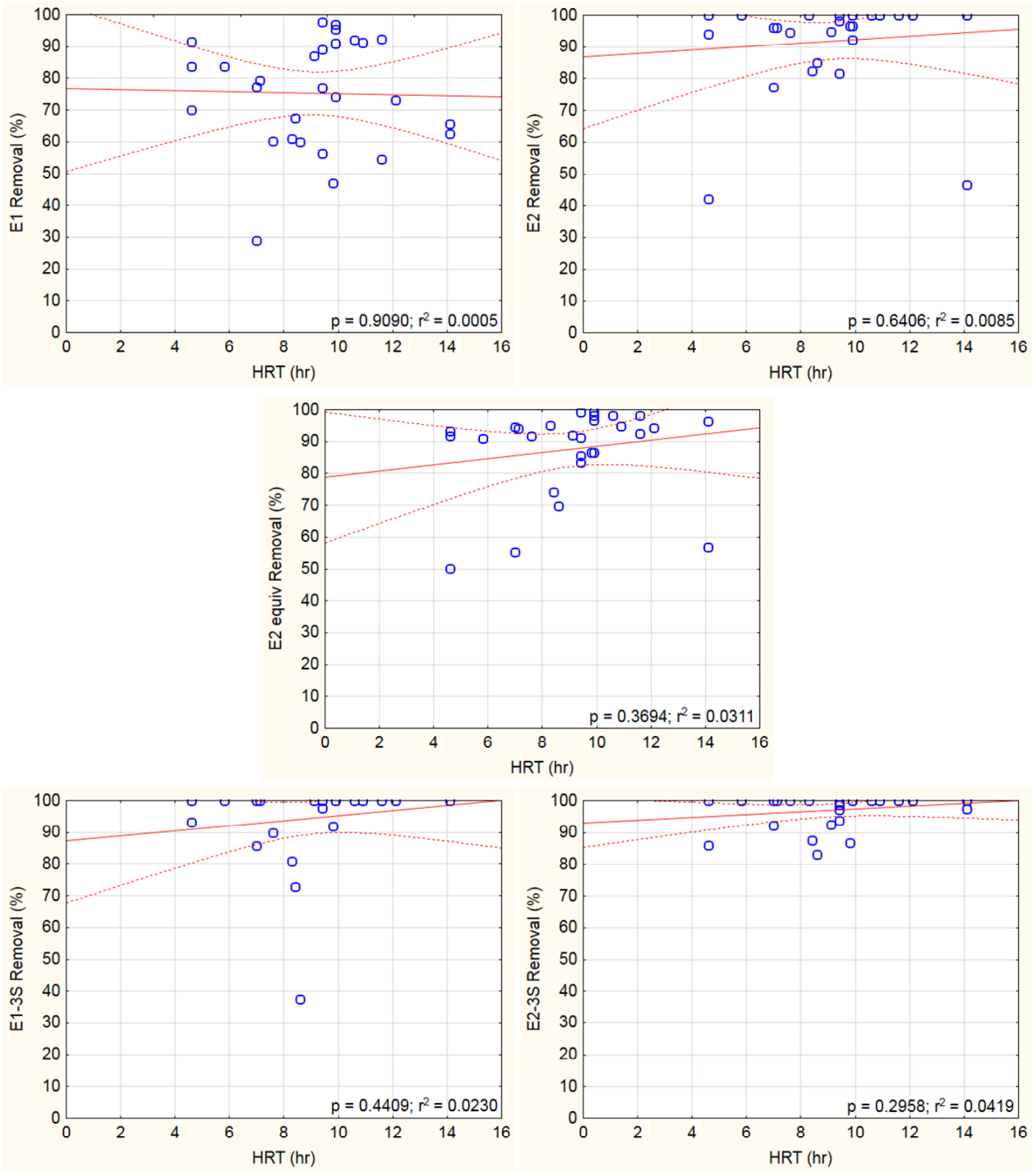

Figure 3 Correlation plot of HRT against the E1, E2, E2 equiv, E1-3S and E2-3S removal for 12 Japanese STPs (n=28).