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Evaluation of attenuation of pharmaceuticals, toxic potency, and antibiotic resistance genes in constructed wetlands treating wastewater effluents

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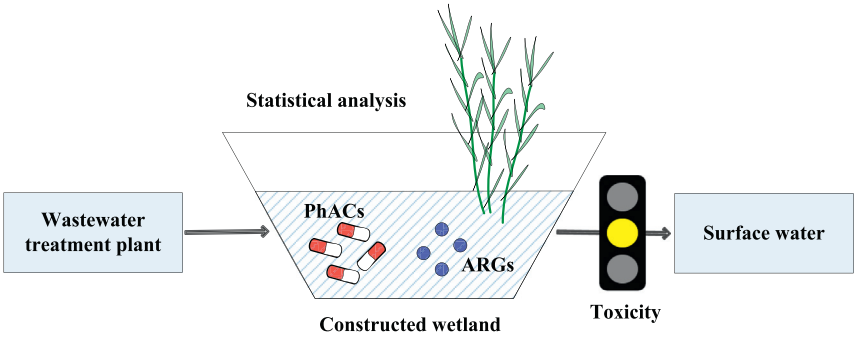
HIGHLIGHTS

- CW performance evaluated by chemical, toxicological and molecular analyses
- Toxic potency assessed by bioanalyses with different varieties of organisms
- Estrogenicity and ARGs were studied for hormones and antibiotics of specific concern.
- Most PhACs related positively to toxic potency and ARGs by multivariate analysis.
- Wastewater effluent discharging to the surface water is of environmental concern.

GRAPHICAL ABSTRACT



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ABSTRACT

The performance of constructed wetlands (CWs) in the removal of pharmaceutically active compounds (PhACs) is generally evaluated on the basis of chemical analysis. In this work, we used a combination of chemical, toxicological, and molecular analyses to assess the attenuation of PhACs, toxic potency and antibiotic resistance genes (ARGs) in a field study of three CWs serving as tertiary treatment of wastewater treatment plants. First, 17 PhACs were analysed chemically, of which 14 were detected and seven at concentrations >0.1 μg/l. Even though some of the individual PhACs were moderately or highly removed in the CWs investigated, median removal of overall PhACs was approximately 50% in the vertical subsurface flow CW (VSF-CW) with a lower hydraulic loading rate while the removal in the other two free water surface flow CWs (SF-CWs) was negligible. Second, toxic potency of wastewater extracts was assessed in a range of bioassays. Estrogenicity was overall attenuated in CWs, while the neurotoxic potency of wastewater extracts did not decrease after passage through the two CWs investigated. Third, the VSF-CW and one of the SF-CW showed a positive removal of an integrase gene and three ARGs tested. The increased concentrations of ARGs in the other SF-CW, as well as the increase of total bacteria in all CWs, may relate to regrowth of resistance-carrying bacteria. Finally, multivariate analysis shows that most PhACs are positively correlated to the observed toxic potency. Additionally, low removal of organics and nutrients seems to parallel with low removal of PhACs. ARGs positively correlated with organics, nutrients and some

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PhACs, and the integrase gene but not to the respective antibiotics. The insufficient removal of PhACs, toxic potency, and ARGs indicates the need of an optimal design of CWs as tertiary treatment facilities.

1. Introduction

The occurrence of pharmaceutically active compounds (PhACs) in the environment is a growing concern due to their potential threat to the aquatic environment and human health. The term PhACs encompasses a diverse group of compounds, such as antibiotics, hormones, analgesic and anti-inflammatory drugs, β-blockers, lipids regulator agents, and antiepileptic drugs (Liu and Wong, 2013). Tons of PhACs are consumed on a global scale (Sebastine and Wakeman, 2003). For example, although annual consumption in the European Union is generally lower than other areas, nonetheless approximately 15,000 tons of human antibiotics are released yearly into the EU environment (Van Boeckel et al., 2014). Due to manufacturing processes, improper disposal and metabolic excretion, PhACs are continuously released into the aquatic environment and as a result exhibit a pseudo-persistent behaviour (Hernando et al., 2006).

PhACs are originally made to elicit a biological effect in target organisms (Henschel et al., 1997). However, with the continuous release of PhACs in the aqueous environment, non-target organisms are and have been exposed over many species generations, which is raising concern towards adverse developmental effects in aquatic ecosystems (Fatta-Kassinos et al., 2011). Among the diverse pool of PhACs, antibiotics are of particular concern (Hernando et al., 2006) as they may accelerate the development of antibiotic resistance genes (ARGs) in microorganisms. This may compromise the effectiveness of antibiotics in curing diseases in humans and live-stock (Kemper, 2008).

Wastewater treatment plants (WWTPs) are the key barrier against the release of PhACs and ARGs to the aquatic environment. However, the conventional treatment processes in WWTPs are not specifically designed for removing PhACs and ARGs, and they are not readily or fully removed (Munir et al., 2011; Verlicchi et al., 2012). To further remove PhACs and ARGs from wastewater effluents, tertiary treatment processes are required. From an economic and environmental perspective, a constructed wetland (CW) could be a promising tertiary treatment technique for removing PhACs and ARGs, as various studies have shown at lab-scale (Hussain et al., 2012; Liu et al., 2013; Matamoros et al., 2008; Zhang et al., 2012), and at field-scale (Chen et al., 2015a, 2015b, 2015c; Conkle et al., 2008; Hijosa-Valsero et al., 2011; Matamoros et al., 2009).

To date, the performance of CWs related to PhAC removal is in most studies only evaluated on the basis of chemical analysis. However, it is nearly impossible to monitor all PhACs and candidate intermediates in all treatment situations, which is further complicated by complex effects of wastewater matrices on analytics of concentrations in the nano- or microgram per liter level. Furthermore, chemical analysis alone gives limited information to understand the potential effects of PhACs and intermediates on the aquatic environment (Välitalo et al., 2016). Bioanalyses employing different test organisms can directly characterize the toxic potency of known and unknown components in a mixed wastewater matrix (Yu et al., 2014). To date, such combined chemical and toxicological studies for CWs are limited and no clear correlation has yet been established between those two analyses.

The emergence of ARGs in humans and animals has been confirmed to correlate significantly with antibiotic use (Wu et al., 2015). However, correlations between the antibiotic abundance and the ARG levels in the environment are uncertain (Wu et al., 2015; Xu et al., 2016). Additionally, antibiotic resistance selection might not only occur through antibiotic selective pressure but also through other chemical pollution such as biocides, heavy metals and detergents (Alonso et al., 2001; Martínez, 2008). Therefore, correlations between ARG levels and the abundance of PhACs including antibiotics in the environment need to be further investigated.

In the work reported here, we conducted a single sampling campaign and assessed the attenuation performance of CWs on 17 PhACs, toxic potency, and three ARGs based on a combination of chemical, toxicological, and molecular analyses. Three full-scale operating CWs were selected, including two free water surface flow CWs (SF-CWs) and one vertical subsurface flow CW (VSF-CW). Bioanalyses were conducted by employing different varieties of receptors, varying from enzymes to algae. The objectives of this study are to 1) identify the level of PhACs, toxic potency and presence of ARGs in wastewater effluents and their attenuation in different types of CWs; 2) explore the correlations between levels of PhACs and toxic potency, and between levels of PhACs and ARGs.

2. Materials and methods

2.1. Chemicals and reagents

Target PhACs were selected from different categories (Table S1 in the supplementary materials), including ketoprofen, diclofenac, ibuprofen, naproxen, erythromycin, lincomycin, sulfamethoxazole, propranolol, metoprolol, clofibric acid, carbamazepine, caffeine, bisphenol A, estrone, 17β-estradiol, ethinylestradiol and estriol, which were purchased from Sigma Aldrich Chemie B·V (the Netherlands). Properties of the target PhACs are shown in Table S1. Other chemicals and reagents used are described in the Text S1.

2.2. Sampling

Wastewater samples were collected in July 2015 from three CWs acting as tertiary treatment process of three WWTPs: Land van Cuijk (L), Hapert (H), and Kaatsheuvel (K) in the Netherlands. Detailed background information of related WWTPs and CWs are shown in Table 1. July was selected as the sampling month due to its low precipitation among the warm months (Fig. S1). CW-L and CW-H are the SF-CWs while CW-K is a VSF-CW.

In order to evaluate the attenuation of PhACs, toxic potency, and ARGs in CWs and the discharge effect of residual PhACs to the following aquatic system, duplicate grab samples were collected at locations indicated in Fig. 1. The attenuation performance of three CWs were calculated based on the difference between CW influent and effluent which is represented by L1–L2, H2–H4, and K1–K2, respectively. It should be noted that the single sampling campaign executed in this study may limit the evaluation of CW attenuation. Possible dilution or concentration of compounds can have occurred depending on the amount of rainwater received. Water samples were collected in 500 ml brown glass bottles, transferred to the laboratory the same day, and stored at 4 °C. Glass bottles for PhAC analysis were pre-washed with ethanol and deionized water, and air dried. For ARG analysis, glass bottles were further autoclaved at 121 °C for 20 min and capped until sampling. Pretreatment of samples (as described in 2.3) was completed within 48 h before being analysed. Wastewater characteristics were detected: dissolved oxygen (DO), pH, and temperature were analysed using a multi-parameter digital meter (Hach HQ40d, USA); chemical oxygen demand (COD), ammonium (NH_4-N), nitrate (NO_3-N), and total phosphate (TP) were analysed by using commercial test kits (Dr. Lange, Hach Lange GmbH, Germany) on a Hach DR/3900 spectrophotometer.

Table 1
Overview and operational parameters of target WWTPs and their CWs.

Parameters	Land van Cuijk (CW-L)	Hapert (CW-H)	Kaatsheuvel (CW-K)
Capacity (inhabitant equivalent)	175,000	71,000	57,300
Wastewater source	43% domestic, 57% industrial[a] & hospital[b]	78% domestic, 22% industrial[c]	Domestic
Biological treatment	Activated sludge with sand filter	Oxidation ditch	Oxidation ditch with sand filter
Flow rate of WWTPs (m^3/h)	2500	718	2200
Effluent treated by CWs	Approx. 25%	Approx. 15%	Approx.10%
Type of CWs	Surface flow, since 1999	Surface flow, since 2001	Vertical subsurface flow, since 1997
Area of CWs (m^2)	20,000	7009	7800
Flow rate of CWs (m^3/h)	360	300	58
Hydraulic retention time of CWs (d)	4	0.82	1.7
Hydraulic loading rate of CWs (cm/d)	43.3	102.7	17.6
Plant species	*Phragmites australis*	*Phragmites australis* (reed bed); trees (swamp)	*Phragmites australis*
Receiving water	River Maas	River Grote Beerze	Lake Ven west

[a] Sources of industrial wastewater are industrial process water, organic biodegradable wastewater, and paper manufacturing wastewater.
[b] Two small hospitals are connected to the sewage system of WWTP Land van Cuijk. The exact individual percentage of industries and hospitals is unknown.
[c] Main sources of industrial wastewater are from meat processing industries, metal industries, and food industries.

2.3. Sample pre-treatment

For chemical and toxicological analyses, samples were pre-treated by filtration (0.7 μm glass filters, GF/F, Whatman, USA) and solid phase extraction (SPE), as previously described (de Wilt et al., 2018). The pre-treatment procedure is described in detail in Text S2 and Fig. S2. In general, 400 ml of samples was loaded on the SPE cartridges to obtain 12 ml elute, of which 3 ml elute was evaporated to achieve a final 500 μl extract with 10% methanol for chemical analysis while 9 ml elute to 500 μl dimethyl sulfoxide (DMSO) for bioanalysis. During SPE, 17β-estradiol-d3 and 10,11-dihydrocarbamazepine were added as the internal standards for gas chromatography tandem mass spectrometry (GC–MS/MS) and ultra-high-performance liquid chromatography (UHPLC)-MS/MS analysis, respectively. Recovery of individual PhACs was tested by spiking PhACs in two different matrices, deionized water and wastewater effluent collected from Bennekom WWTP, the Netherlands. Recovery rates are summarized in Table S2. As recovery of diclofenac was low from wastewater, concentration of diclofenac was determined by direct injection on UHPLC-MS/MS after being pre-treated by centrifugation at 10,000 rpm for 10 min (Microlite, Thermo IEC, USA).

For ARG analysis, 500 ml water samples were filtered using 0.2 μm membrane filter (Merck Milipore, Ireland) and the filter was placed in centrifuge tubes. Those tubes were stored at −20 °C before DNA extraction.

2.4. Chemical and bioanalyses

Hormones were analysed on a GC–MS/MS and quantification of other PhACs was performed by a UHPLC-MS/MS. Detailed analytical methods are shown in Text S3, Table S3, and Table S4.

Bioanalyses to quantify the toxic potency were performed by using 96-wells plates and detected by a plate reader (Tecan infinite M200 PRO, Switzerland). Different receptors, including yeast, green algae, acetylcholinesterase (AchE) and luminescence bacteria were exposed to the wastewater extracts to determine their acute and chronic toxic potency (Table S5). The AchE assay quantifies the potency of the compounds present to inhibit the acetylcholine esterase enzyme, a measure of neurotoxic potency. The REA (RIKILT Estrogen Assay) with the human estrogen receptor α (hERα), comparable to the yeast estrogen screening assay, was performed to quantify the estrogenicity specifically for the hERα. The microtiter Microtox assay provides measure of general toxic and the microtiter algal growth inhibition assay (AGIA) is a measure of the phytotoxicity of the hydrophilic compounds present in the wastewater extracts. Methods were validated by reference compounds (Fig. S3) and the responses expressed relative to that of assay-specific standards. DMSO was used as the blank control. The emission and excitation spectra of wastewater extract were scanned (Fig. S4) to make sure that the background of wastewater did not overlap with selected measurement wavelengths in Table S5. The wastewater extracts were tested in quintuplicate in microtiter AGIA and in triplicate for the other bioanalyses. More information about the bioanalysis protocols is shown in Text S4.

For all the conducted bioanalyses, results were reported in inhibition percentage as well as toxic equivalence concentrations for that assay (TEQs, Eqs. (1)–(3)), which is the concentration of a reference compound used to elicit the same response as the unknown and undefined mixture of compounds actually present (Macova et al., 2010).

$$\mathrm{TEQ}_s = \frac{\mathrm{EC}_{50}(\text{reference compound})}{\mathrm{EC}_{50}(\text{sample})} = \frac{C_{\text{reference compound}} \times \text{dilution factor}_{\text{reference compoud}}}{\text{enrichment factor}_{\mathrm{SPE}} \times \text{dilution factor}_{\text{sample}}} \quad (1)$$

$$\text{Dilution factor}_{\text{reference compound}} = \frac{\text{volume of reference compound added to the bioassay}}{\text{total volume of the bioassay}} \quad (2)$$

$$\text{Enrichment factor}_{\mathrm{SPE}} = \frac{\text{volume of sample loading in SPE}}{\text{volume of extract}} \quad (3)$$

$C_{\text{reference compound}}$ is the 50% effective concentration (EC_{50}) of reference compound added to the wells. In principle, any effect level can be used to derive TEQs other than EC_{50}, provided that the concentration-effect curves are reliable (Villeneuve et al., 2000). In this study, dilution factor of reference compound was the same with that of wastewater extracts in each bioanalysis. Therefore, the TEQs of the extracts ($TEQ_{extract}$) are the ratios of $C_{reference\ compound}$ and Enrichment $factor_{SPE}$ (600 times).

2.5. DNA extraction and ARG quantification

DNA filters of water samples were processed using a PowerWater DNA Isolation Kit (MoBio Laboratories, USA), according the manufacturer's protocols. The extracted DNA was stored at −80 °C until further analysis.

Quantitative PCR (qPCR) was used to quantify the abundances of 16S-rRNA, the integrase gene *Intl1* and three ARGs, including *sul1* and *sul2* (sulfonamide resistance genes), and *ermB* (macrolide resistance gene). These genes have been recommended for environmental monitoring of antibiotic resistance (Berendonk et al., 2015; Gillings et al., 2015) and have been detected in wastewater at high prevalence and concentrations (Chen et al., 2015c; Rodriguez-Mozaz et al., 2015), making them suitable for analysis of attenuation efficiencies. A synthetic standard with a known quantity was used as the standard for each

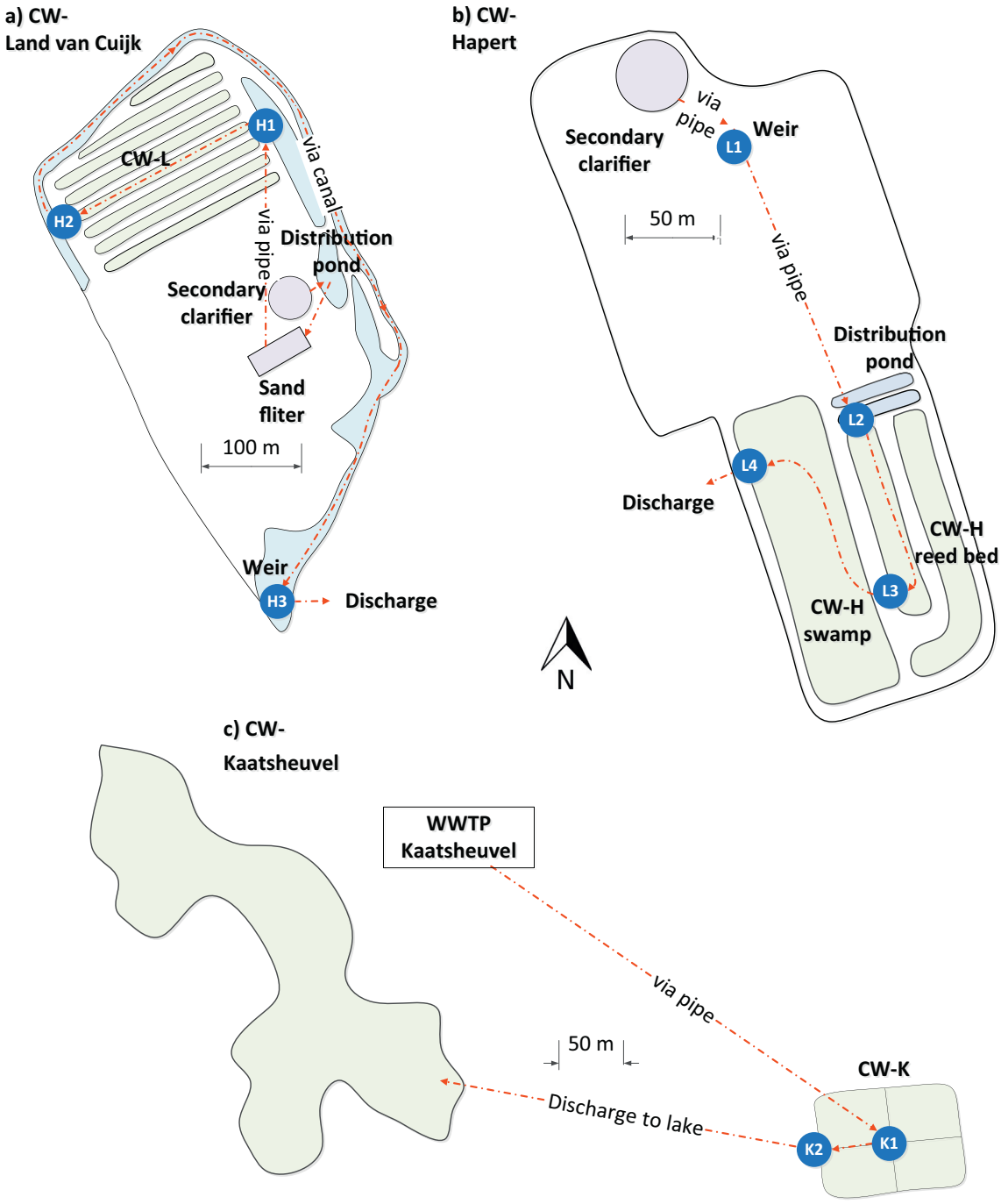

Fig. 1. Sampling points in three constructed wetlands (CWs) a) Land van Cuijk (L), L1: inlet of CW; L2: outlet of CW; L3: discharge point; (b) Hapert (H); H1: effluent of secondary clarifier; H2: inlet of CW (after distribution pond); H3: after reed bed treatment; H4: discharge point to the surface water (after swamp treatment); (c) Kaatsheuvel (K). K1: effluent of sand filter/inlet of CW; K2: outlet of CW. Dash lines only indicate the flow direction rather than the real flow paths. The figure was drawn based on the geographical map.

gene and DNase free water was used as the blank control. Samples, standards and blanks were run using the same procedure in duplicate. Samples were diluted 50 times to avoid qPCR inhibition by humic acids, biological contaminants or proteins. qPCR assays were conducted using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, USA) and iQ™ Supermix (Bio-Rad Laboratories, USA) for the SYBR Green reactions and TaqMan reactions, respectively. The reaction mixture of 10 μl consisted of our sample, master mix, primers (Eurogentec, Belgium), precision blue (Biorad, USA), DNase- and RNase-free water. Details of qPCR conditions and primers are shown in Table S6. qPCR assays were conducted on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Canada). Abundances of genes were normalized to 16S-rRNA values to represent the resistance genes per total bacteria. Detection limit is in the range of 4.81×10^0–4.70×10^7 copies/ml for 16S-rRNA, *Intl1* and all ARGs.

2.6. Statistical analysis

In order to investigate the correlation between removal of PhACs and their physicochemical properties, principal component analysis in Section 3.2 was conducted by using SIMCA 13.0. In addition, multivariate analysis in Section 3.5 was conducted using the CANOCO 5 software package (Biometrics, the Netherlands) to analyze the correlation 1) among wastewater characteristics, concentration of PhACs and

related toxic potency; and 2) among wastewater characteristics, the abundance of PhACs and ARGs. Response variables in multivariate analysis were centralized and standardized to achieve zero average and unit variance. ARG data was processed by BioRad CFX Manager 3.1 (Biorad, USA).

3. Results and discussion

3.1. Wastewater characteristics

The wastewater macro-chemical characteristics DO, pH, temperature, COD, NH_4-N, NO_3-N, and TP, as well as removal efficiencies of these by the CWs are presented in Table S7. Discharged wastewater of all three CWs complied with the urban wastewater treatment standards (Council Directive 91/271/EEC). CWs showed a capacity to remove nutrients, but this removal was incomplete: CW-L removed 66% of NO_3-N and 21% of TP; CW-H removed 23% of NH_4-N; CW-K removed 26% of COD and 37% of NO_3-N (Table S7). Levels of NO_3-N and TP in the wastewater stream further decreased with 66% and 21% after passing the canal and ponds (L2 to L3 in CW-L), indicating that open waters could attenuate nutrients as well.

3.2. PhACs

Of the 17 target PhACs, only the lipid regulator clofibric acid and the estrogenic compounds ethinylestradiol and estriol, were not detected in the WWTP effluent before entering the CWs, and estrone and 17β-estradiol only in a few samples (Table 2). It cannot be excluded that this was because the samples were not deconjugated before analysis, as (synthetic) hormones are excreted as conjugates (Legler et al., 2002) and as such not detected by the chemical analysis. Ketoprofen only was detected in CW-H. Seven out of 14 detected PhACs discharged to the surface water were at concentrations higher than the guidance value of 0.1 μg/l adopted by toxicologists for drinking water (Verliefde et al., 2007). No regulations exist yet for PhACs discharged from WWTPs to the surface water. Concentrations of ibuprofen (5.0–6.5 μg/l) and bisphenol A (0.9–3.7 μg/l) even reached the microgram per liter level. The abundance of ibuprofen was also shown by the study of Verlicchi and Zambello (2014), in which ibuprofen was found to be the most abundant PhAC out of 137 PhACs in 136 different CW systems with an average of 32 μg/l and 9.8 μg/l in the influent and effluent of horizontal subsurface flow CWs (HSF-CWs), respectively. Bisphenol A levels were higher in CW-L and CW-H than in CW-K. The latter only receives wastewater from households while the other two CWs also receive wastewater from industries as well (Table S2). We assumed that part of the detected bisphenol A in CW-L and CW-H might come from the industries, considering bisphenol A is an industrial compound manufactured in large quantities as a monomer for producing polycarbonate and epoxy resins (Im and Löffler, 2016).

Some PhACs were detected at higher levels in the subsequent sampling point compared to the upstream point closer to the WTTP effluent discharge point, which might have originated from the single sampling campaign or be caused by deconjugation. A number of compounds are excreted in the conjugated form. It has been reported that many PhACs that are excreted by people as glucuronides or other conjugated metabolites can be converted back to the parent compound by enzymatic processes (Breitholtz et al., 2012; Sun et al., 2008). Deconjugation has been observed in WWTPs occasionally for antibiotics, hormones, and psychiatric drugs (Gros et al., 2010; He et al., 2013; Vieno et al., 2007).

Among the detected 14 PhACs, erythromycin, sulfamethoxazole, propranolol, and metoprolol could be highly removed (>75%) in the compartments of the CWs; diclofenac, naproxen, and lincomycin were removed moderately (30%–60%); while there was almost no removal or even an increase for the other compounds such as bisphenol A (Table S8). Fate of PhACs was reported to be correlated with their physicochemical properties (Lee et al., 2011). In our study, principal component analysis was conducted to investigate the relationships between removal efficiencies of PhACs and combination of their properties including the dissociation constant pK_a, the octanol–water partition coefficient log K_{ow} and log D_{ow}. Log D_{ow} is the apparent partitioning coefficient considering the combined effect of pK_a and log K_{ow}. The results showed no correlation between PhACs removal and those properties (Fig. S5). The non-correlation with pK_a and log K_{ow} is also in line with previous research, which proved that PhACs behavior in the biological treatment processes cannot be determined by a certain chemical parameter alone (Breitholtz et al., 2012; Park et al., 2009; Zhang et al., 2012). The absence of correlation with a single or a combination of physical parameters indicates that more removal mechanism (e.g. phytoremediation) might be involved other than biodegradation and adsorption in our case.

Removal efficiencies of 14 detected PhACs were evaluated in three CWs and open waters. Strikingly, the median removal of PhACs in CW-K was approximately 50% while that in CW-L and CW-H (both

Table 2
Concentrations of target pharmaceutically active compounds (PhACs) in different sampling points (average ± std, $n = 2$).

Category	PhACs	Concentration (ng/l)								
		Land van Cuijk (CW-L)			Hapert (CW-H)				Kaatsheuvel (CW-K)	
		L1	L2	L3	H1	H2	H3	H4	K1	K2
Anti-inflammatory drugs	Ketoprofen	<7	<7	<7	16 ± 11	17 ± 8	17 ± 8	18 ± 5	<7	<7
	Diclofenac	38 ± 13	25 ± 4	22 ± 1	82 ± 8	71 ± 6	78 ± 1	65 ± 5	46 ± 2	21 ± 5
	Ibuprofen	5319 ± 346	5905 ± 1132	3691 ± 702	6492 ± 261	4325 ± 295	5266 ± 510	5770 ± 292	4971 ± 130	4584 ± 486
	Naproxen	355 ± 5	211 ± 25	307 ± 40	180 ± 17	181 ± 14	174 ± 0	173 ± 3	174 ± 4	74 ± 1
Antibiotics	Erythromycin	19 ± 0	20 ± 1	21 ± 2	42 ± 0	45 ± 4	47 ± 9	42 ± 4	45 ± 4	3 ± 1
	Lincomycin	4 ± 0	5 ± 0	5 ± 0	7 ± 0	7 ± 1	7 ± 0	42 ± 1	4 ± 0	3 ± 0
	Sulfamethoxazole	74 ± 4	70 ± 4	55 ± 3	362 ± 8	378 ± 74	447 ± 9	330 ± 4	179 ± 4	43 ± 2
Beta-blockers	Propranolol	41 ± 2	26 ± 14	13 ± 0	58 ± 10	67 ± 27	79 ± 7	68 ± 1	61 ± 1	11 ± 6
	Metoprolol	434 ± 15	536 ± 23	123 ± 0	1426 ± 35	1407 ± 16	1454 ± 1	1334 ± 8	1114 ± 3	168 ± 12
Lipid regulators	Clofibric acid	<10								
Psychiatric drugs	Carbamazepine	230 ± 11	226 ± 7	227 ± 5	204 ± 7	211 ± 0	218 ± 1	216 ± 4	248 ± 1	316 ± 1
Stimulants	Caffeine	123 ± 14	142 ± 13	70 ± 6	118 ± 22	130 ± 2	141 ± 7	132 ± 2	133 ± 10	207 ± 48
Estrogenic compounds	Bisphenol A	1232 ± 1107	1593 ± 69	1995 ± 1215	3682 ± 1847	2033 ± 728	2722 ± 1020	3828 ± 806	870 ± 319	1441 ± 63
	Estrone	ND	3 ± 2	3 ± 0	3 ± 0	ND	ND	ND	ND	ND
	17β-Estradiol	ND	ND	ND	ND	45 ± 4	53 ± 15	76 ± 4	ND	ND
	Ethinylestradiol	<10								
	Estriol	<10								

reed bed and swamp compartment) was negligible (Fig. 2). The removal efficiencies we observed are relatively low compared with previous studies where removal of most investigated PhACs in CWs were higher than 50% (Hijosa-Valsero et al., 2011; Matamoros et al., 2009). The better PhAC removal performance of CW-K might be caused by its vertical configuration and operational parameters such as hydraulic loading rate (HLR). On the one hand, CW-K is a VSF-CW while the other two CWs are SF-CWs. Compared with SF-CWs, VSF-CWs usually achieve a better oxygenation and possess a superior rhizosphere effect in rhizodegradation as well as adsorption (Matamoros et al., 2007; Zhang et al., 2014). In fact, Matamoros et al. (Matamoros et al., 2009) reported that >50% of the studied PhACs were better and more consistently removed in the VSF-CWs as compared with other technologies such as compact biofilters and biological sand filters. On the other hand, a lower HLR in CWs was reported to result in a higher removal of PhACs due to longer contact and interaction among nutrients, substrate and roots (Ávila et al., 2014; Zhang et al., 2012). In our study, CW-K with a lower HLR (Table 1) indeed showed higher removal for PhACs.

PhACs could be attenuated in open waters (L2 to L3, H1 to H2 in Fig. 2), in which PhACs are directly exposed to the sunlight. In fact, PhAC removal has been verified in ponds, either as polishing ponds followed by CWs or as tertiary treatment units in WWTPs, in which photodegradation might play an important role (Hijosa-Valsero et al., 2010; Matamoros and Salvadó, 2012; Rühmland et al., 2015). Therefore, CWs are suggested to include shallow open water compartments to enhance photodegradation of PhACs. But still, CWs with plants are useful as they are rich in biomass and thus are less affected by seasonal changes for removing biodegradable PhACs compared with ponds (Matamoros and Salvadó, 2012).

3.3. Toxic potency

In the present study, toxic potency of wastewater extracts was assessed by five bioanalyses based on different receptors. Results are expressed as inhibition or response to receptors and relative to that of the reference standard (toxic equivalence concentrations, TEQs for that assay). In the microtiter AGIA and Microtox assays no toxicity was observed (Fig. S6). Interestingly, the vitality of the algae and bacteria was even enhanced when wastewater extracts were added, possibly because the extracts contained nutrients. It was confirmed that this was not due to background color or fluorescence from the extracts. The same enhancement phenomenon was also found in previous studies (He et al., 2016; Lundström et al., 2010).

In the YTA, toxic potency of wastewater extracts was notably attenuated in CW-H but not in the other two CWs (Fig. 3a). The retained toxic potency reflected from TEQ YTA is of 0.85–3.33 μg tributyltin-EQ/l. The AchE inhibiting potency was reduced in CW-L, while the other two CWs had no capacity to attenuate this neurotoxic potency (Fig. 3b). The TEQ_{AChE} was in the range of 6.4–11.8 μg dichlorvos-EQ/l (DEQ) for surface water discharging points. This level of dichlorvos is exceeding the maximum permissible concentration of 0.7 ng/l in Dutch surface waters (Crommentuijn et al., 1997) and the 50% lethal concentration (LC50) of 190 ng/l for Daphnia (Hamers et al., 2001). This shows that the potential aquatic risks in the wastewater are not negligible.

The estrogenic potency of the sample extracts, as analysed in the REA, decreased overall in sequential operational compartments, from

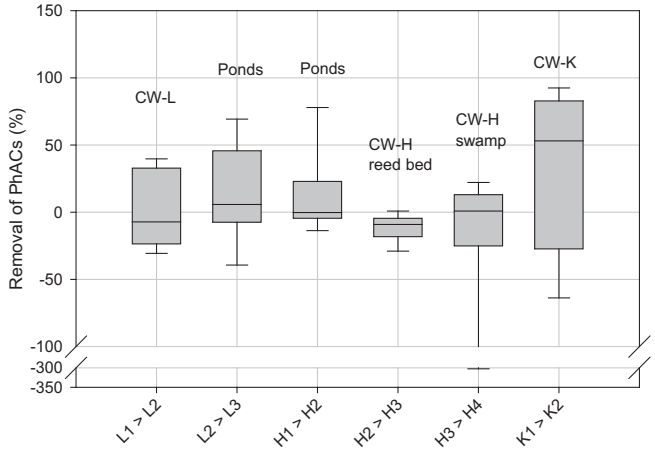

Fig. 2. Removal efficiencies of 14 detected pharmaceutically active compounds (PhACs) in constructed wetlands (CWs) and open waters. The box plot shows the values in maximum, third quartile, median, first quartile, and minimum.

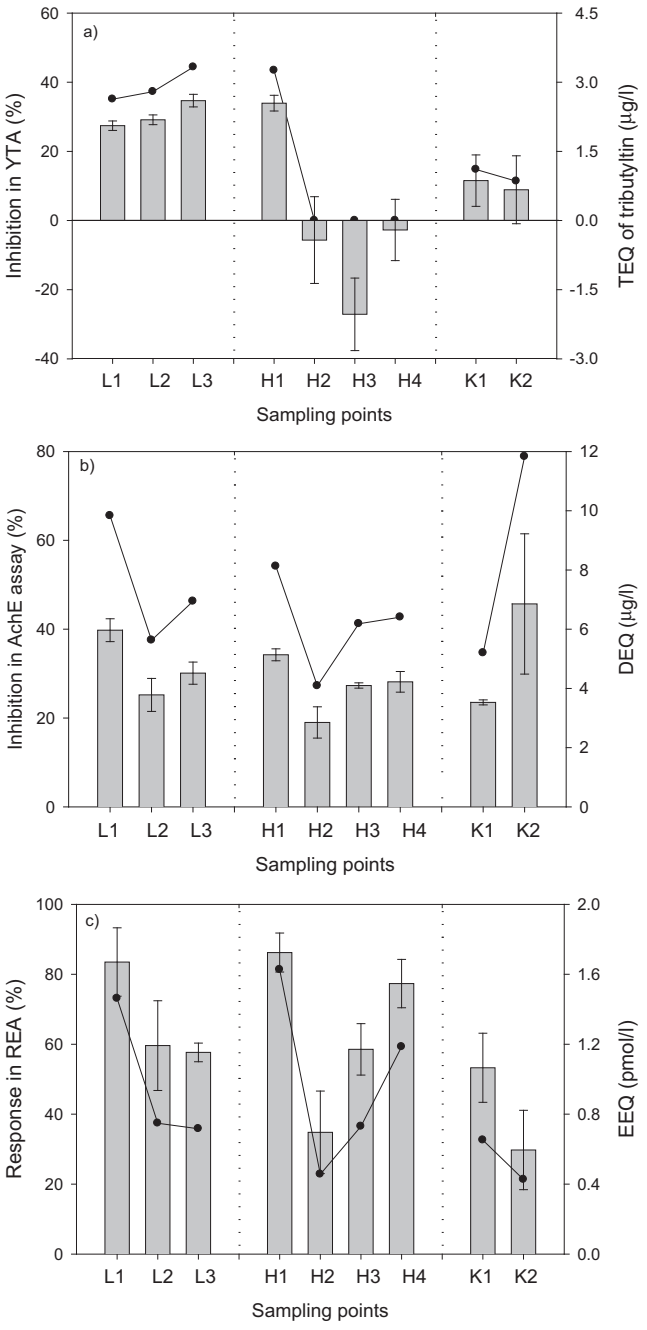

Fig. 3. Toxic potency and toxic equivalence concentrations (TEQs) of wastewater extracts in various compartment positions in the CW for three different bioanalyses: a) yeast toxicity assay (YTA); b) acetylcholinesterase (AChE) assay; c) RIKILT Estrogen Assay (REA). The left axis corresponds to data in bars; the right axis corresponds to data in dots. Toxicity data are mean value ± standard error ($n = 6$).

point L1 to L3, H1 to H2, and K1 to K2 (Fig. 3b). Only in H3 and H4 the potency increased, which happens to be the only locations in which 17β-estradiol was found with the chemical analysis. The observed 17β-estradiol may originate from additional sources such as hormones from the massive inhabitant birds that we visually observed. The same assumption was also previously reported for CW-H in the work of Foekema (2012). In our study, the level of estradiol equivalents (EEQ) was 0.4–1.6 pmol/l (Fig. 3b), which is of the same magnitude as the EEQ detected in Dutch wastewater effluent in previous studies: 0.9–2.5 pmol/l (Foekema, 2012) and 0–2.1 pmol/l (Murk et al., 2002). Even though the estrogenicity decreased in operational compartments before discharging to surface water bodies, still the observed estrogeniticity level is of concern. A previous study showed that 1.3 pmol/l EEQ could affect immature male rainbow trout to produce estrogen biomarker vitellogenin after 28 weeks of dosing (Sheahan et al., 1994).

In summary, wastewater extracts showed in general toxicity in YTA and specific toxicity in REA and AChE assays. The DEQ levels as determined in the AChE assay are above the LC50 for Daphnia and above the maximum permissible concentration for dichlorvos, and therefore are of environmental concern. No attenuation of the toxic potency in the AchE assay was observed after passage through the two CWs investigated.

3.4. Antibiotic resistant genes

In the present study, an integrase gene (*intl1*) and three ARGs (*sul1*, *sul2*, *and ermB*) were investigated in the wastewater. All ARGs were detected in the wastewater samples except that *ermB* was under detection limit at K2 (Fig. 4a). Overall, the class 1 integron gene *intl1* had the highest concentrations. Among ARGs, the abundance of *sul1* was highest followed by *sul2* and *ermB*, in terms of both absolute concentrations and concentrations relative to the total bacterial community. The detected ARG concentrations varied from 4.9 copies/ml (*ermB*, L2) to 1.7×10^5 copies/ml (*sul1*, H4) in wastewater samples (Fig. 4a). The findings in this study are in line with previous studies in which *sul1* and *sul2* genes were the most abundant ARGs in CWs (Chen et al., 2015c; Chen et al., 2016), rivers (Chen et al., 2015b; LaPara et al., 2015; Proia et al., 2016), and marine environments (Suzuki et al., 2013).

CW-L and CW-K showed positive removal of the absolute concentrations of all ARGs in the range of 14% (*Intl1*) to 95% (*sul2*), and 57% (*Intl1*) to almost 100% (*ermB*) (Table S9). Meanwhile, CW-H showed negative removal (i.e. increase) of all ARGs except for *ermB* (70%). Notably, the total bacteria increased in all the investigated CWs (Table S9). Relative to the total bacterial, most resistance genes remained stable or showed a decrease after CW treatment (L1–L2, H2–H4, K1–K2, Fig. 4b). Some of the previous researches concluded that CWs are able to reduce the

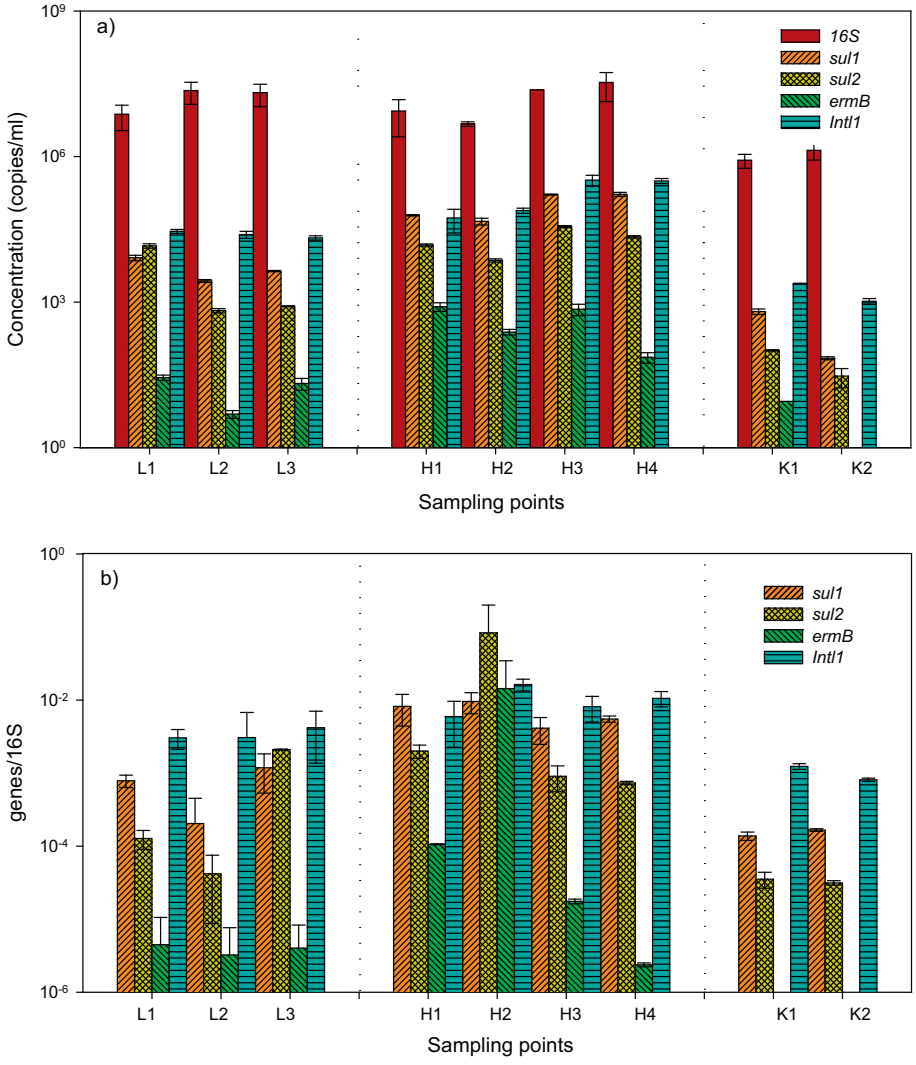

Fig. 4. The a) absolute concentrations and b) normalized concentrations of genes in three constructed wetlands CW-L, CW-H and CW-K. Results are mean value ± standard deviation ($n = 2$).

concentration of ARGs (Auerbach et al., 2007; Chen et al., 2016; Fang et al., 2017), while some also observed a significant increase in antibiotic resistance after CW treatment, either in absolute concentrations (Nõlvak et al., 2013) or relative concentrations (Huang et al., 2015; Liu et al., 2013). Suitable conditions can promote regrowth of microorganisms after the treatment (Zhang et al., 2016), which might lead to the observed absolute increases of ARGs. In addition, selective pressures might be present that also promote regrowth of resistance-carrying bacteria, including antibiotic selective pressure which might be present even at low concentrations (Gullberg et al., 2011; Tello et al., 2012), interaction mediated by antibiotics or non-antibiotic metabolites (Bernier and Surette, 2013), or heavy metal selective pressure (Baker-Austin et al., 2006).

3.5. Multivariate analysis of PhACs, toxic potency, and ARGs data

Multivariate analysis was conducted to explore chemical, toxicological and molecular outcomes, and their correlation was investigated through projections onto the ordinations obtained. The detected toxic potency is positively correlated to wastewater characteristics and PhACs (Fig. 5a). Various researchers have positively correlated organics (COD) and nutrients to the toxic potency of wastewater (Bayo et al., 2009; Ma et al., 2016; Yu et al., 2014). In contrast, PhACs have rarely been correlated with toxic potency in real wastewater matrixes. In our study, toxic potency described in REA and YTA seemed to be positively correlated with wastewater characteristics and PhACs. Especially REA positively correlated to organics, nutrients (NH_4-N, NO_3-N, TP) as well as most of the PhACs. In comparison, AchE correlated less with environmental variables, indicating that the neurotoxic potency of wastewater extracts might be related to other pollutants than the PhACs we tested.

ARG levels are not correlated to the abundance of related antibiotics but rather to organics, nutrients, and some PhACs (Fig. 5b). Higher concentrations of SMZ and ETM did not correlate with higher concentrations of *sul1/2* and *ermB*, respectively. This lack of correlation may result from three reasons: 1) wastewater already contains high amounts of resistance genes, which are not necessary related to the actual wastewater antibiotic content; 2) abundance of resistance gene in the CWs result from survival (or even growth) of wastewater bacteria carrying these genes, or selection of resistant bacteria in situ. These processes are in turn possibly partly, but not exclusively mediated by PhACs or other selective pressures; 3) mobile genetic element such as plasmids, integrases, and transposases are able to assist the spread of ARGs without antibiotics being present (Zhu et al., 2013). Thus, antibiotics and resistance genes do not necessarily have to be correlated, as also shown in previous research (Anderson et al., 2013; Pruden et al., 2006; Wu et al., 2015). However, a positive correlation was found between concentrations of ARGs and concentrations of organics, nutrients and some PhACs. This might indicate that organics and nutrients stimulate growth of resistant bacteria in CWs, and that processes removing these pollutants also reduce resistance genes to a similar extent.

Sul1, *sul2* and *ermB* show strong correlations with *intl1* (Fig. 5b), indicating that removal or regrowth of bacteria harboring these genes in general co-occurs. The *intl1* gene has been found to be correlated with the dissemination of both types of *sul* genes in the environment (Chen et al., 2015a). The *sul1* gene is normally found in class 1 integrons *intl1* (Sköld, 2000), whereas *sul2* is usually located on small non-conjugative plasmids (Enne et al., 2001) or large transmissible, multi-resistance plasmids (Heuer and Smalla, 2007). In a study of Antunes et al. (2005), they observed *intl1* presence in almost 98% of *sul1* isolates. Shehabi et al. (2006) also found that 62% of *sul1*/*sul2* was positively associated with *intl1*. With this correlation, Muziasari et al. (2014) suggested that *intl1* may play a role in the prevalence of *sul1* through horizontal gene transfer.

As indicated in Fig. 5a and b, the observed positive correlation between concentrations of organics and nutrients and concentrations of PhACs shows that conditions that remove organics and nutrients most likely remove PhACs as well. Similar results were found in the study of Matamoros et al. (2007), in which the authors positively linked the high removal of most targeted PhACs with the high removal of BOD, total suspended solid (TSS), and NH_4-N in a VSF-CW and sand filter systems. Therefore, the low removal of organics and nutrients might explain the low attenuation of PhACs in this study.

Although the single sampling campaign in this work may limit the evaluation of CW attenuation performance, the multivariate analysis implemented in this study provides more insight into the presence and removal of PhACs as well as their associated environmental hazards (i.e. toxic potency and ARGs). These results overall show a snapshot of

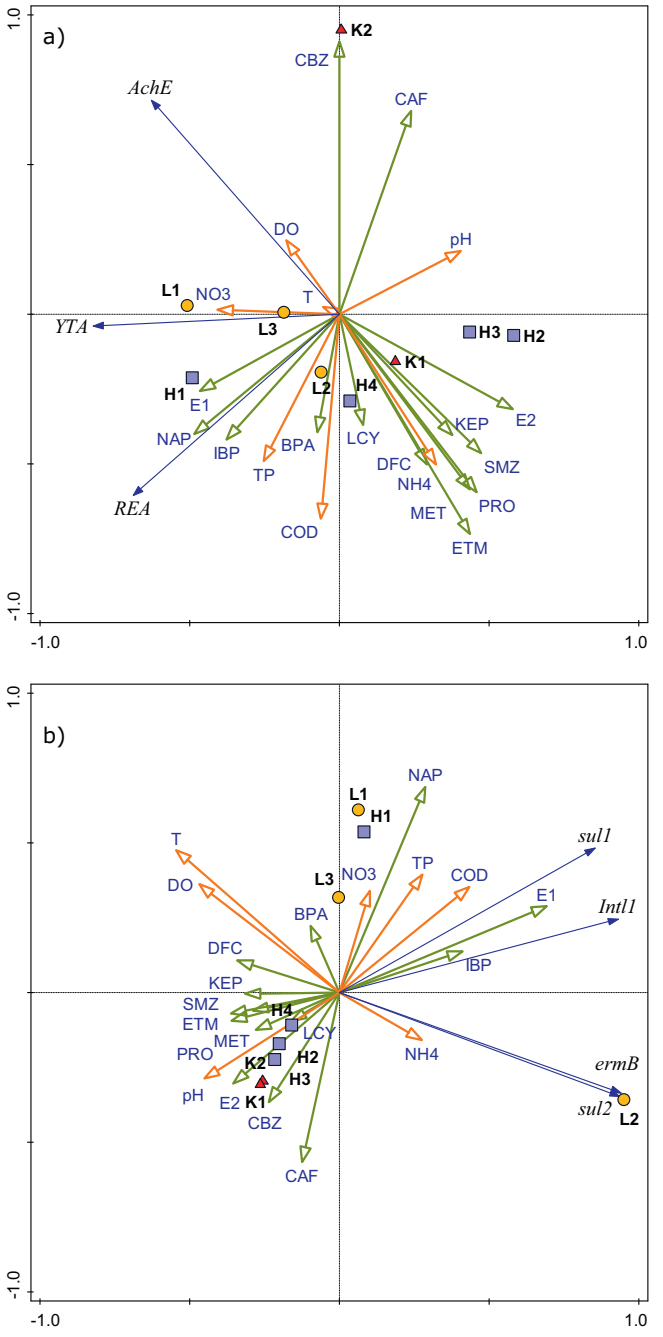

Fig. 5. Multivariate analysis of the correlation a) among toxic potency, wastewater characteristics and PhACs; b) among ARGs (normalized), wastewater characteristics and PhACs. Sampling points are indicated in circles for CW-L, rectangles for CW-H, and triangles for CW-K, respectively. Environmental variables are shown in arrows. The eigenvalues of the first and second canonical axis are 0.51 and 0.29 in a), 0.84 and 0.13 in b).

limited and variable attenuation of PhACs, toxic potency and ARGs in the three CWs. The findings might indicate many removal processes in the CWs are sub-optimal and more knowledge generation on the attenuation mechanisms under varying CWs operational conditions is essential. More repeated measurement should be conducted in the future to confirm this indication.

4. Conclusions

In this study, performance of CWs to attenuate PhACs, toxic potency, and ARGs has been assessed. Furthermore, correlations between toxic potency, PhACs, ARGs, and water characteristics were explored. The main findings are: 1) Several PhACs discharged to the surface water were at concentrations higher than 0.1 μg/l, especially for bisphenol A and ibuprofen. Even though some of the PhACs were moderately or highly removed, the median removal of PhACs in CWs was approximate 50% in CW-K and negligible in other two CWs. 2) Wastewater extracts showed general toxicity in YTA and specific toxicity in REA and AChE assays. The DEQ levels are above safe levels and therefore are of environmental concern. In addition, the DEQ levels did not show attenuation in two of the CWs investigated. 3) Positive ARG removal was observed in CW-L and CW-K in terms of both absolute and relative concentrations. The increased absolute concentrations of *sul1*, *sul2*, and *Intl1* in CW-H as wells as the increase of total bacteria in all CWs may link to regrowth of microorganisms mediated by suitable growth conditions and/or selective pressures. 4) Most PhACs were positively correlated to the toxic potency, either indicating a potential hazard of these compounds to the environment or indicating co-occurrence of PhAC with other substances in the wastewater causing toxic potency. Concentrations of organics, nutrients, and some PhACs were positively correlated to ARG concentrations while no concrete pattern of ARGs can be predicted from the concentration of the antibiotics analysed.

Considering the insufficient removal of PhACs, toxic potency and ARGs in CWs, enhancement of CW performance is desirable, where optimal construction (e.g. vertical configuration, constructed with open waters) and operational parameters (e.g. HLR) can be considered. Multivariate analysis in this study offers a great potential to comprehensively evaluate the performance of CWs by associating chemical, toxicological, and molecular analyses.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.03.083.

References

Alonso, A., Sánchez, P., Martínez, J.L., 2001. Environmental selection of antibiotic resistance genes. Environ. Microbiol. 3 (1), 1–9.

Anderson, J., Carlson, J., Low, J., Challis, J., Wong, C., Knapp, C., Hanson, M., 2013. Performance of a constructed wetland in Grand Marais, Manitoba, Canada: removal of nutrients, pharmaceuticals, and antibiotic resistance genes from municipal wastewater. Chemistry Central Journal 7, 54.

Antunes, P., Machado, J., Sousa, J.C., Peixe, L., 2005. Dissemination of sulfonamide resistance genes (*sul1*, *sul2*, *and sul3*) in Portuguese *Salmonella enterica* strains and relation with integrons. Antimicrob. Agents Chemother. 49, 836–839.

Auerbach, E.A., Seyfried, E.E., McMahon, K.D., 2007. Tetracycline resistance genes in activated sludge wastewater treatment plants. Water Res. 41, 1143–1151.

Ávila, C., Matamoros, V., Reyes-Contreras, C., Piña, B., Casado, M., Mita, L., Rivetti, C., Barata, C., García, J., Bayona, J.M., 2014. Attenuation of emerging organic contaminants in a hybrid constructed wetland system under different hydraulic loading rates and their associated toxicological effects in wastewater. Sci. Total Environ. 470–471, 1272–1280.

Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J.V., 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. 14, 176–182.

Bayo, J., Angosto, J.M., Gómez-López, M.D., 2009. Ecotoxicological screening of reclaimed disinfected wastewater by *Vibrio fischeri* bioassay after a chlorination–dechlorination process. J. Hazard. Mater. 172, 166–171.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Burgmann, H., Sorum, H., Norstrom, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317.

Bernier, S., Surette, M., 2013. Concentration-dependent activity of antibiotics in natural environments. Front. Microbiol. 4, 1–14.

Breitholtz, M., Näslund, M., Ståe, D., Borg, H., Grabic, R., Fick, J., 2012. An evaluation of free water surface wetlands as tertiary sewage water treatment of micro-pollutants. Ecotoxicol. Environ. Saf. 78, 63–71.

Chen, B., Hao, L., Guo, X., Wang, N., Ye, B., 2015b. Prevalence of antibiotic resistance genes of wastewater and surface water in livestock farms of Jiangsu Province, China. Environ. Sci. Pollut. Res. 1–10.

Chen, B., Liang, X., Nie, X., Huang, X., Zou, S., Li, X., 2015a. The role of class I integrons in the dissemination of sulfonamide resistance genes in the Pearl River and Pearl River Estuary, South China. J. Hazard. Mater. 282, 61–67.

Chen, J., Liu, Y.S., Su, H.C., Ying, G.G., Liu, F., Liu, S.S., He, L.Y., Chen, Z.F., Yang, Y.Q., Chen, F.R., 2015c. Removal of antibiotics and antibiotic resistance genes in rural wastewater by an integrated constructed wetland. Environ. Sci. Pollut. Res. 22, 1794–1803.

Chen, J., Wei, X.D., Liu, Y.S., Ying, G.G., Liu, S.S., He, L.Y., Su, H.C., Hu, L.X., Chen, F.R., Yang, Y.Q., 2016. Removal of antibiotics and antibiotic resistance genes from domestic sewage by constructed wetlands: optimization of wetland substrates and hydraulic loading. Sci. Total Environ. 565, 240–248.

Conkle, J.L., White, J.R., Metcalfe, C.D., 2008. Reduction of pharmaceutically active compounds by a lagoon wetland wastewater treatment system in Southeast Louisiana. Chemosphere 73, 1741–1748.

Crommentuijn, T., Kalf, D., Polder, M., Posthumus, R., 1997. Maximum Permissible Concentrations and Negligible Concentrations for Pesticides.

de Wilt, A., He, Y., Sutton, N., Langenhoff, A., Rijnaarts, H., 2018. Sorption and biodegradation of six pharmaceutically active compounds under four different redox conditions. Chemosphere 193, 811–819.

Enne, V.I., Livermore, D.M., Stephens, P., Hall, L.M.C., 2001. Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. Lancet 357, 1325–1328.

Fang, H., Zhang, Q., Nie, X., Chen, B., Xiao, Y., Zhou, Q., Liao, W., Liang, X., 2017. Occurrence and elimination of antibiotic resistance genes in a long-term operation integrated surface flow constructed wetland. Chemosphere 173, 99–106.

Fatta-Kassinos, D., Meric, S., Nikolaou, A., 2011. Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. Anal. Bioanal. Chem. 399, 251–275.

Foekema, E., 2012. De Invloed van Moerassystemen op de Milieukwaliteit van Rwzi Effluent en Aanbevelingen tot Optimalisering. IMARES rapport C005/12.

Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.-G., 2015. Using the Class 1 Integron-integrase Gene as a Proxy for Anthropogenic Pollution. Vol. 9 pp. 1269–1279.

Gros, M., Petrović, M., Ginebreda, A., Barceló, D., 2010. Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environ. Int. 36, 15–26.

Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D., Andersson, D.I., 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 7, e1002158.

Hamers, T., Smit, M.G.D., Murk, A.J., Koeman, J.H., 2001. Biological and chemical analysis of the toxic potency of pesticides in rainwater. Chemosphere 45, 609–624.

He, Y., Chen, W., Zheng, X., Wang, X., Huang, X., 2013. Fate and removal of typical pharmaceuticals and personal care products by three different treatment processes. Sci. Total Environ. 447, 248–254.

He, Y., Sutton, N.B., Rijnaarts, H.H.M., Langenhoff, A.A.M., 2016. Degradation of pharmaceuticals in wastewater using immobilized TiO_2 photocatalysis under simulated solar irradiation. Appl. Catal., B 182, 132–141.

Henschel, K.P., Wenzel, A., Diedrich, M., Fliedner, A., 1997. Environmental hazard assessment of pharmaceuticals. Regul. Toxicol. Pharmacol. 25, 220–225.

Hernando, M.D., Mezcua, M., Fernández-Alba, A.R., Barceló, D., 2006. Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta 69, 334–342.

Heuer, H., Smalla, K., 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ. Microbiol. 9, 657–666.

Hijosa-Valsero, M., Matamoros, V., Martín-Villacorta, J., Bécares, E., Bayona, J.M., 2010. Assessment of full-scale natural systems for the removal of PPCPs from wastewater in small communities. Water Res. 44, 1429–1439.

Hijosa-Valsero, M., Matamoros, V., Sidrach-Cardona, R., Pedescoll, A., Martín-Villacorta, J., García, J., Bayona, J.M., Bécares, E., 2011. Influence of design, physico-chemical and environmental parameters on pharmaceuticals and fragrances removal by constructed wetlands. Water Sci. Technol. 63, 2527–2534.

Huang, X., Liu, C., Li, K., Su, J., Zhu, G., Liu, L., 2015. Performance of vertical up-flow constructed wetlands on swine wastewater containing tetracyclines and tet genes. Water Res. 70, 109–117.
Hussain, S.A., Prasher, S.O., Patel, R.M., 2012. Removal of ionophoric antibiotics in free water surface constructed wetlands. Ecol. Eng. 41, 13–21.
Im, J., Löffler, F.E., 2016. Fate of bisphenol A in terrestrial and aquatic environments. Environ. Sci. Technol. 50, 8403–8416.
Kemper, N., 2008. Veterinary antibiotics in the aquatic and terrestrial environment. Ecol. Indic. 8, 1–13.
LaPara, T.M., Madson, M., Borchardt, S., Lang, K.S., Johnson, T.J., 2015. Multiple discharges of treated municipal wastewater have a small effect on the quantities of numerous antibiotic resistance determinants in the upper Mississippi River. Environ. Sci. Technol. 49, 11509–11515.
Lee, S., Kang, S.-I., Lim, J.-L., Huh, Y.J., Kim, K.-S., Cho, J., 2011. Evaluating controllability of pharmaceuticals and metabolites in biologically engineered processes, using corresponding octanol–water distribution coefficient. Ecol. Eng. 37, 1595–1600.
Legler, J., Jonas, A., Lahr, J., Vethaak, A.D., Brouwer, A., Murk, A.J., 2002. Biological measurement of estrogenic activity in urine and bile conjugates with the in vitro ER-CALUX reporter gene assay. Environ. Toxicol. Chem. 21, 473–479.
Liu, J., Wong, M., 2013. Pharmaceuticals and personal care products (PPCPs): a review on environmental contamination in China. Environ. Int. 59, 208–224.
Liu, L., Liu, C., Zheng, J., Huang, X., Wang, Z., Liu, Y., Zhu, G., 2013. Elimination of veterinary antibiotics and antibiotic resistance genes from swine wastewater in the vertical flow constructed wetlands. Chemosphere 91, 1088–1093.
Lundström, E., Adolfsson-Erici, M., Alsberg, T., Björlenius, B., Eklund, B., Lavén, M., Breitholtz, M., 2010. Characterization of additional sewage treatment technologies: ecotoxicological effects and levels of selected pharmaceuticals, hormones and endocrine disruptors. Ecotoxicol. Environ. Saf. 73, 1612–1619.
Ma, K., Qin, Z., Zhao, Z., Zhao, C., Liang, S., 2016. Toxicity evaluation of wastewater collected at different treatment stages from a pharmaceutical industrial park wastewater treatment plant. Chemosphere 158, 163–170.
Macova, M., Escher, B.I., Reungoat, J., Carswell, S., Chue, K.L., Keller, J., Mueller, J.F., 2010. Monitoring the biological activity of micropollutants during advanced wastewater treatment with ozonation and activated carbon filtration. Water Res. 44, 477–492.
Martínez, J.L., 2008. Antibiotics and antibiotic resistance genes in natural environments. Science 321 (5887), 365–367.
Matamoros, V., Arias, C., Brix, H., Bayona, J.M., 2007. Removal of pharmaceuticals and personal care products (PPCPs) from urban wastewater in a pilot vertical flow constructed wetland and a sand filter. Environ. Sci. Technol. 41, 8171–8177.
Matamoros, V., Arias, C., Brix, H., Bayona, J.M., 2009. Preliminary screening of small-scale domestic wastewater treatment systems for removal of pharmaceutical and personal care products. Water Res. 43, 55–62.
Matamoros, V., Caselles-Osorio, A., García, J., Bayona, J.M., 2008. Behaviour of pharmaceutical products and biodegradation intermediates in horizontal subsurface flow constructed wetland. A microcosm experiment. Sci. Total Environ. 394, 171–176.
Matamoros, V., Salvadó, V., 2012. Evaluation of the seasonal performance of a water reclamation pond-constructed wetland system for removing emerging contaminants. Chemosphere 86, 111–117.
Munir, M., Wong, K., Xagoraraki, I., 2011. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Res. 45, 681–693.
Murk, A., Vethaak, A., Rijs, G., Schrap, S., Ruiter, H., Gerritsen, A., Lahr, J., 2002. Estrogens and Xeno-estrogens in the Aquatic Environment of The Netherlands: Occurence, Potency and Biological Effects. Rijkswaterstaat, RIKZ.
Muziasari, W.I., Managaki, S., Pärnänen, K., Karkman, A., Lyra, C., Tamminen, M., Suzuki, S., Virta, M., 2014. Sulphonamide and trimethoprim resistance genes persist in sediments at Baltic Sea aquaculture farms but are not detected in the surrounding environment. PLoS One 9, e92702.
Nõlvak, H., Truu, M., Tiirik, K., Oopkaup, K., Sildvee, T., Kaasik, A., Mander, Ü., Truu, J., 2013. Dynamics of antibiotic resistance genes and their relationships with system treatment efficiency in a horizontal subsurface flow constructed wetland. Sci. Total Environ. 461, 636–644.
Park, N., Vanderford, B.J., Snyder, S.A., Sarp, S., Kim, S.D., Cho, J., 2009. Effective controls of micropollutants included in wastewater effluent using constructed wetlands under anoxic condition. Ecol. Eng. 35, 418–423.
Proia, L., von Schiller, D., Sànchez-Melsió, A., Sabater, S., Borrego, C.M., Rodríguez-Mozaz, S., Balcázar, J.L., 2016. Occurrence and persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers. Environ. Pollut. 210, 121–128.
Pruden, A., Pei, R., Storteboom, H., Carlson, K.H., 2006. Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. Environ. Sci. Technol. 40, 7445–7450.
Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D., Balcázar, J.L., 2015. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 69, 234–242.
Rühmland, S., Wick, A., Ternes, T.A., Barjenbruch, M., 2015. Fate of pharmaceuticals in a subsurface flow constructed wetland and two ponds. Ecol. Eng. 80, 125–139.
Sebastine, I.M., Wakeman, R.J., 2003. Consumption and environmental hazards of pharmaceutical substances in the UK. Process. Saf. Environ. Prot. 81, 229–235.
Sheahan, D., Bucke, D., Matthiessen, P., Sumpter, J., Kirby, M., Neall, P., Waldock, M., 1994. The Effects of Low Levels of 17a-Ethynylestradiol upon Plasma Vitellogenin Levels in Male and Female Rainbow Trout, *Oncorhynchus mykiss* Held at Two Acclimation Temperatures.
Shehabi, A., Odeh, J., Fayyad, M., 2006. Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from human stools and drinking water sources in Jordan. J. Chemother. 18, 468–472.
Sköld, O., 2000. Sulfonamide resistance: mechanisms and trends. Drug Resist. Updat. 3, 155–160.
Sun, Q., Deng, S., Huang, J., Shen, G., Yu, G., 2008. Contributors to estrogenic activity in wastewater from a large wastewater treatment plant in Beijing, China. Environ. Toxicol. Pharmacol. 25, 20–26.
Suzuki, S., Ogo, M., Miller, T., Shimizu, A., Takada, H., Siringan, M.A., 2013. Who possesses drug resistance genes in the aquatic environment?: sulfamethoxazole (SMX) resistance genes among the bacterial community in water environment of Metro-Manila, Philippines. Front. Microbiol. 4.
Tello, A., Austin, B., Telfer, T.C., 2012. Selective pressure of antibiotic pollution on bacteria of importance to public health. Environ. Health Perspect. 120, 1100–1106.
Välitalo, P., Perkola, N., Seiler, T.-B., Sillanpää, M., Kuckelkorn, J., Mikola, A., Hollert, H., Schultz, E., 2016. Estrogenic activity in Finnish municipal wastewater effluents. Water Res. 88, 740–749.
Van Boeckel, T.P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B.T., Levin, S.A., Laxminarayan, R., 2014. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. Lancet Infect. Dis. 14, 742–750.
Verlicchi, P., Al Aukidy, M., Zambello, E., 2012. Occurrence of pharmaceutical compounds in urban wastewater: removal, mass load and environmental risk after a secondary treatment—a review. Sci. Total Environ. 429, 123–155.
Verlicchi, P., Zambello, E., 2014. How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? A review. Sci. Total Environ. 470–471, 1281–1306.
Verliefde, A., Cornelissen, E., Amy, G., Van der Bruggen, B., van Dijk, H., 2007. Priority organic micropollutants in water sources in Flanders and the Netherlands and assessment of removal possibilities with nanofiltration. Environ. Pollut. 146, 281–289.
Vieno, N., Tuhkanen, T., Kronberg, L., 2007. Elimination of pharmaceuticals in sewage treatment plants in Finland. Water Res. 41, 1001–1012.
Villeneuve, D.L., Blankenship, A.L., Giesy, J.P., 2000. Derivation and application of relative potency estimates based on in vitro bioassay results. Environ. Toxicol. Chem. 19, 2835–2843.
Wu, D., Huang, Z., Yang, K., Graham, D., Xie, B., 2015. Relationships between antibiotics and antibiotic resistance gene levels in municipal solid waste leachates in Shanghai, China. Environ. Sci. Technol. 49, 4122–4128.
Xu, Y., Guo, C., Luo, Y., Lv, J., Zhang, Y., Lin, H., Wang, L., Xu, J., 2016. Occurrence and distribution of antibiotics, antibiotic resistance genes in the urban rivers in Beijing, China. Environ. Pollut. 213, 833–840.
Yu, X., Zuo, J., Tang, X., Li, R., Li, Z., Zhang, F., 2014. Toxicity evaluation of pharmaceutical wastewaters using the alga *Scenedesmus obliquus* and the bacterium *Vibrio fischeri*. J. Hazard. Mater. 266, 68–74.
Zhang, D.Q., Gersberg, R.M., Hua, T., Zhu, J.F., Tuan, N.A., Tan, S.K., 2012. Pharmaceutical removal in tropical subsurface flow constructed wetlands at varying hydraulic loading rates. Chemosphere 87, 273–277.
Zhang, D.Q., Gersberg, R.M., Ng, W.J., Tan, S.K., 2014. Removal of pharmaceuticals and personal care products in aquatic plant-based systems: a review. Environ. Pollut. 184, 620–639.
Zhang, S., Lin, W., Yu, X., 2016. Effects of full-scale advanced water treatment on antibiotic resistance genes in the Yangtze Delta area in China. FEMS Microbiol. Ecol. 92, 1–8.
Zhu, Y.-G., Johnson, T.A., Su, J.-Q., Qiao, M., Guo, G.-X., Stedtfeld, R.D., Hashsham, S.A., Tiedje, J.M., 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc. Natl. Acad. Sci. 110, 3435–3440.