Science of the Total Environment 636 (2018) 464–476

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Identification, contribution, and estrogenic activity of potential EDCs in a river receiving concentrated livestock effluent in Southern Taiwan

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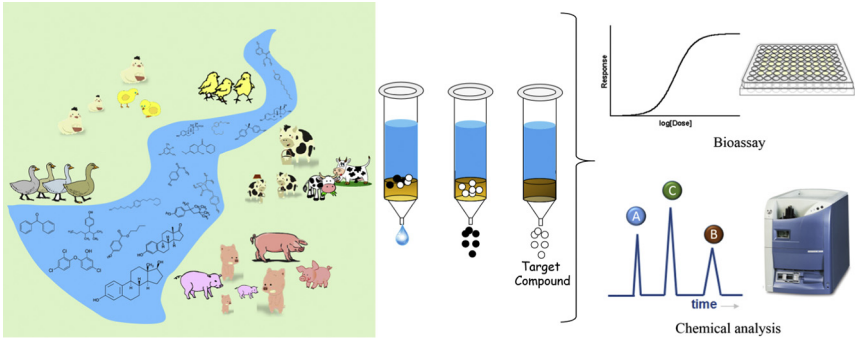
HIGHLIGHTS

- The presence of 22 emerging compounds was determined for an area containing intensive animal feedlot operations.
- Estrogenic activity obtained from T47D-Kbluc assays on real samples was at much lower levels in pre-concentration extracts.
- Highest contributions to estrogenic activity were due to the presence of estrone and 17ß-estradiol.
- Estrogenic activity being underestimated may be due to conjugated estrogens or their metabolites being excluded.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 31 January 2018
Received in revised form 2 April 2018
Accepted 3 April 2018
Available online xxxx

Keywords:
Livestock discharge
EDCs
T47D-KBluc
MCF-7 bioassays
Alkylphenolic compounds
LC-MS/MS
E1 and E2 levels

ABSTRACT

We assessed 22 selected endocrine-disrupting compounds (EDCs) and other emerging, potentially endocrine-active compounds with estrogenic activity from the waters of the Wuluo River, southern Taiwan. This watershed receives high amounts of livestock and untreated household wastewaters. The river is surrounded by concentrated animal feedlot operations (CAFOs). River water samples were analyzed for selected compounds by liquid chromatography-tandem mass spectrometry (LC-MS/MS), T47D-KBluc reporter gene assay, and E-screen cell proliferation *in vitro* bioassay. Total concentrations of ∑alkylphenolic compounds (bisphenol A, 4-nonylphenol, t-nonylphenol, octylphenol, nonylphenol mono-ethoxylate, nonylphenol di-ethoxylate) were much higher than ∑estrogens (estrone, 17 β-estradiol, estriol, 17ß-ethynylestradiol, diethylstilbestrol), ∑preservatives (methyl paraben, ethyl paraben, propyl paraben, butyl paraben), ∑UV-filters (benzophenone, methyl benzylidene camphor, benzophenone-3), ∑antimicrobials (triclocarben, triclosan, chloroxylenol), and an insect repellent (diethyltoluamide) over four seasonal sampling periods. The highest concentration was found for bisphenol A with a mean of 302 ng/L. However, its contribution to estrogenic activity was not significant due to its relatively low estrogenic potency. Lower detection rates were found for BP, EE2, TCS, and PCMX, while DES and EP were not detected. E1 and E2 levels in raw water samples were 50% higher than the predicted no-effect concentrations (PNEC) for aquatic organisms of 6 and 2 ng/L, respectively. The potency of estrogenic activity ranged from 11.7 to 190.1 ng/L $E2_{T47D\text{-}Kbluc}$ and 6.63 to 84.5 ng/L $E2_{E\text{-}Screen}$ for extracted samples. Importantly, estrone contributed 50% of the overall activity in 60% and 44% of the samples based on T47D-KBluc and MCF-7 bioassays, followed by 17 ß-estradiol, highlighting the importance of total steroid estrogen loading. This study

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https://doi.org/10.1016/j.scitotenv.2018.04.031

demonstrates that the estrogenic activity of target chemicals was comparable to levels found in different countries worldwide. More intense wastewater treatment is required in areas of intensive agriculture in order to prevent adverse impacts on the ambient environment and aquatic ecosystems.

1. Introduction

There are huge amounts of chemical substances in use, some of which impact the endocrine system and are often classified as endocrine disrupting chemicals (EDCs). Studies show that EDCs are a serious problem due to their presence in many daily necessities such as plastic products, flame retardants, food additives, toys, detergents, and personal care products such as sunscreens and cosmetics (Colborn et al., 1993; Markey et al., 2002; Mezcua et al., 2012; Yang et al., 2015). Personal care products, food, and beverages contain such EDCs as natural phytoestrogens or pesticides. EDCs have a similar structure to hormones, particularly those of invertebrates and humans. They might interfere with endocrine functions even at very low concentrations (Diamanti-Kandarakis et al., 2009; Schug et al., 2011). There is evidence that these effects may not only affect exposed individuals but also have adverse multi-generational effects (Borysko and Ross, 2014). The effects of natural, synthetic, or xenoestrogenic compounds on aquatic organisms by these EDCs, as well as simulation models for endocrine functioning, are receiving increased attention (Ying et al., 2002). However, due to the high costs of chemical analysis for a variety of emergent or suspected toxic compounds and their lack of bioeffect information, *in vitro* assays were developed as risk assessment tools. The yeast estrogen screen assay (YES-assay), T47D-KBluc reporter gene assay, E-Screen cell proliferation assay, and ER-CALUX luciferase gene expression bioassay are examples of fast molecular screening tools. These assays have become potential alternatives to *in vivo* testing due to their ability to detect overall estrogenic activities contributed by known and unknown compounds within an environmental sample.

Endocrine disrupting chemicals, in addition to natural and synthetic hormones, preservatives, UV filters and other suspected xenoestrogens, contribute to total estrogenic activity in whole effluents and urgently warrant further discussion.

1.1. Natural and synthetic estrogens

Different reproductively active EDCs affecting the environment and humans can be derived from natural estrogens (estrone (E1), 17ß-estradiol (E2), and estriol (E3)) and synthetic estrogenic compounds (17α-ethynylestrodiol (EE2) and diethylstilbestrol (DES)). These have attracted attention, and E1, E2, E3, and EE2 are currently listed in the USEPA's Drinking Water Contaminant Candidate List 4 (Ying et al., 2002; USEPA, 2016).

E2 (17β-estradiol) is the main estrogenic compound from animal discharges (particularly fish and land vertebrates) and is associated with the maintenance of animal reproductive systems and sexual characteristics (Welshons et al., 2003). E2 is mainly excreted through urine and feces, with excretions varying with sex, age, fitness, and generally with physiological and developmental status. The main estrogen contributors are pregnant or menstruating women (Barreiros et al., 2016). The U.S. Department of Health and Human Services pointed out that E2 may be a carcinogen since it is associated with increased testicular, prostate, and breast cancers (US Department of Health and Human Services, 1994). Another study demonstrated that the E2 metabolite of E3 increased the estrogen response of endocrine responsive (ER) positive breast cancer cells even at a concentration of 10^{-10} M (Diller et al., 2014). The potency of ER combined with E2 is higher than E1 with E3 (Kuiper et al., 1997). EE2 (17α-ethynylestrodiol) is a synthetic estrogen that is mainly used in oral contraceptives, pre-menopausal pharmaceuticals, and the treatment of prostate cancer, breast cancer, osteoporosis, and other diseases (Kuster et al., 2005). Besides being used as drugs in humans, EE2 increases the productivity of livestock and prevents reproductive disorders (Bartelt-Hunt et al., 2011). Physicochemically, EE2 has little solubility and is easily absorbed into the environmental matrix and accumulated in organisms where it causes chronic impacts (De Wit et al., 2010; Chen et al., 2010).

1.2. Preservatives

Parabens (PBs) were developed in the 1920s as antiseptics for pharmaceutical products. PBs were later widely used in food, beverages, and as antimicrobials in industrial products and cosmetics, personal care products, and pesticides (Andersen, 2008). The properties of PBs vary with the length of the chain of alkyl groups (methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP), and butyl paraben (BP)) and in combination are commonly synergistic with enhanced antimicrobial effects. Cosmetics contain one or more PB additives in >13,200 formulations that act as antibacterial agents (Andersen, 1984). The release of PBs into the environment through discharge into water bodies or as granular nanoparticles emitted into the atmosphere contributes to environmental pollution (Błędzka et al., 2014). Studies show that PBs might induce breast cancer or pernicious melanoma, although PBs have a low affinity to estrogen receptors (Darbre et al., 2004; Golden et al., 2005). However, other studies indicate that they change their hormonal properties after extended paraben exposure (Darbre and Harvey, 2008). The Food and Drug Administration (Taiwan) has set out rules that MP used alone or mixture should not be used in higher proportions than 0.4% and should not exceed 0.8% in "Cosmetic Preservative Use and Limitation Reference Value" (TFDA, 2016). The highest allowable limit for BP and PP concentration in food is 0.14% when each is used alone. As an additive, they should total <1% in cosmetics regulated by the Taiwan Food and Drug Administration (TFDA) of the Ministry of Health and Welfare (2016), while other limits are explained in another report (Andersen, 2008).

Ethyl paraben (EP) and butyl paraben (BP) are white crystals or powders that can be added to cosmetics, personal care products, and preservatives in food (Haman et al., 2015; Papadopoulos et al., 2016). Although the endocrine disrupting effects of EP and BP are insignificant, they are commonly detected in a variety of environmental matrices under prolonged usage and insufficient removal by sewage treatment (Haman et al., 2015). *In vitro* bioassays prove that the estrogenic activity of EP is about 1/200,000 of that of estradiol and shows a multiplier effect in MCF-7 cell assays (Miller et al., 2001; Byford et al., 2002). Miller et al. (2001) and Vinggaard et al. (2000) noted weak estrogenic activity from paraben in yeast assays. Ethyl, propyl, and butyl parabens possess estrogen-mimicking properties in fish (Barse et al., 2010; Bjerregaard et al., 2003). Some recent studies have reported adverse reproductive effects from parabens (Boberg et al., 2010; González-Doncel et al., 2014). Okubo et al. (2001) also reported on the estrogenic activity of ethyl-, propyl-, butyl-, isopropyl-, and isobutyl paraben in human MCF-7 breast cancer cells. Darbre et al. (2003) reported estrogenic activity caused by benzyl paraben in human MCF-7 and ZR-75-1 cell lines. Propyl- and butyl paraben have estrogenic potencies comparable to bisphenol A (Pedersen et al., 2000). A related toxicological assessment found that the subcutaneous exposure of BP induced toxic effects on the reproductive system in young male rats, which could affect the fertility of animals (Garcia et al., 2017). In the presence of estradiol,