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# An *in vivo* assay performed using multiple biomarkers related to testosterone synthesis and conversion for assessing the androgenic potency of refuse leachate

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ABSTRACT

Refuse leachate is likely an important source of androgens. However, common *in vitro* bioassays underestimate the potential androgenic activity of leachate, owing to non-receptor-mediated mechanisms that modify the balance of sex hormones and promote the accumulation of endogenous androgens. This study aimed to develop an *in vivo* assay by using multiple biomarkers related to testosterone synthesis and conversion for assessing the potential androgenic activity of refuse leachate sampled from a municipal solid waste treatment plant in Qingdao, China. The results indicated that exposure to leachate increased the levels of testosterone and luteinizing hormone, but decreased those of 17$\beta$-estradiol in both male and female goldfish (*Carassius auratus*), suggesting a potential androgenic activity. Further, Leydig cell hyperplasia and decreased gonadal P450 aromatase mRNA levels were observed; these alterations might promote the biosynthesis of testosterone and hinder the conversion of testosterone to 17$\beta$-estradiol, which in turn enhance testosterone accumulation. Exposure to leachate also resulted in reproductive impairments, including decreased gonadosomatic index and plasma vitellogenin levels of female goldfish, as well as decreased testicular enzyme activities in male goldfish. The integrated use of biochemical, molecular, and histological markers not only improved our understanding of the androgenic effects of leachate but also verified the reliability and validity of the results. Therefore, the *in vivo* bioassay described in this study might allow the investigation of the androgenic effects of other complex contaminant mixtures in the future.



## 1. Introduction

In China, sanitary landfill and incineration have been the most popular ways of disposing solid waste materials. Both methods generate highly contaminated refuse leachate that poses threats to the environment (Kjeldsen et al., 2002; Ye et al., 2011). Due to microbial degradation (Stamps et al., 2016), leachate harbors various endocrine-disrupting chemicals (EDCs) such as bisphenol A (Masoner et al., 2014), phthalates (Ramakrishnan et al., 2015), polybrominated diphenyl ethers (Kiddee et al., 2014), organotin compounds (Mersiowsky et al., 2001), and steroid hormones (Masoner et al., 2016). These chemicals can cause hormonal disorders and reproductive impairments in wild animals (Noaksson et al., 2001, 2003a). Previous studies on endocrine disruptions induced by leachate have mainly focused on its estrogenicity (Behnisch et al., 2001; Kamata et al., 2011). However, information on its potential androgenic activity is limited.

Svenson et al. (2004) used an *in vitro* recombinant yeast strain transfected with human androgen receptor (AR) to assess the androgenicity of leachate samples from Sweden and found a maximum androgenic potency of 900 ng 5α-dihydrotestosterone equivalents per liter. However, in addition to the AR-mediated mechanism, androgenic effects can also be induced *via* the disruption of normal steroidogenesis and increase in the levels of endogenous androgens such as testosterone (T) (Ankley et al., 2002; Hallgren et al., 2006). For example, tributyltin (TBT), a frequently detected compound in leachate, exerts masculinization by suppressing P450 aromatase (P450arom) and increasing T levels in fish (Pinel-Raffaitin et al., 2008; Shimasaki et al., 2003; Vahčič et al., 2011; Zhang et al., 2007). Such non-AR-mediated mechanisms cannot be detected by AR-based *in vitro* bioassays, thereby likely leading to false-negative results or underestimation owing to the complexity of leachate. The use of multiple biomarkers

*Abbreviations:* AR, androgen receptor; T, testosterone; TBT, tributyltin; P450arom, P450 aromatase; GtHs, gonadotropins; LH, luteinizing hormone; FSH, follicle-stimulating hormone; $E_2$, 17$\beta$-estradiol; Vtg, vitellogenin; GSI, gonadosomatic index; $\gamma$-GTP, gamma-glutamyl transpeptidase, ACP, acid phosphatase; ALP, alkaline phosphatase; MBR, membrane bioreactor; DTRO, disc-tube reverse osmosis; RIA, radioimmunoassay; PCR, polymerase chain reaction

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*in vivo* might overcome these drawbacks and serve as a reliable tool for the assessment of the androgenic potential of leachate.

Leydig cells are known to be the major cellular source of androgens (Hoar and Nagahama, 1978); further, the synthesis and conversion of endogenous androgens are known to be regulated by gonadotropins (GtHs) and P450arom. GtHs, including luteinizing hormone (LH) and follicle-stimulating hormone (FSH), regulate steroidogenesis and gametogenesis in fish (Allan et al., 2004; Meachem et al., 2005), whereas P450arom catalyzes the final step in the conversion of T to 17$\beta$-estradiol ($E_2$) (Simpson et al., 1994). All these are excellent biomarkers for assessing the androgenic effects induced by non-AR-mediated mechanisms.

In this study, multiple biochemical, molecular, and histological markers (including plasma T, $E_2$, LH, and FSH levels; testis histology; and gonadal P450arom mRNA levels) in goldfish (*Carassius auratus*) were used as specific biomarkers for evaluating the potential androgenic activity of leachate samples collected from a municipal solid waste treatment plant in Qingdao, China. In addition, since endocrine disruptions are always related to reproductive impairments, the plasma levels of vitellogenin (Vtg); the gonadosomatic index (GSI); and the activity of testicular enzymes, including gamma-glutamyl transpeptidase ($\gamma$-GTP), acid phosphatase (ACP), and alkaline phosphatase (ALP), were analyzed to assess the potential reproductive toxicity of leachate.

## 2. Materials and methods

### 2.1. Municipal solid waste treatment plant

Leachate samples were collected from a municipal solid waste treatment plant located in Qingdao Prefecture, eastern China, in 2013. It has an area of 65.88 ha and has been in operation since 2002. It treats around 3000 t $day^{-1}$ municipal solid waste from the Qingdao city and consists of three parts, *i.e.*, a landfill site, refuse incineration plant, and leachate treatment plant.

### 2.2. Leachate treatment system

The leachate is treated with a membrane bioreactor (MBR) comprising four bioreactors and an external ultrafiltration unit and disc-tube reverse osmosis (DTRO) technology. The capacity of the leachate treatment facility is 900 $m^3$ $d^{-1}$ and the treated effluent discharged into the adjacent river meets Grade one A standard of the *Discharge Standard of Pollutants for Municipal Wastewater Treatment Plant* (GB18918-2002, China). A flow diagram of the leachate treatment system is shown in Fig. 1. Briefly, leachate is first filtered by screen filters to remove large suspended solids and homogenized in a regulating reservoir, which has dimensions of 9.6 m × 6.0 m × 5.0 m, holding about 950 $m^3$ of leachate. Next, the raw leachate is fed to the MBR process. In MBR configurations, leachate passes through a two-stage A/O process involving alternating anoxic and aerobic conditions to remove the organic matter, ammonia nitrogen and nitrate nitrogen. Ultrafiltration membranes (polyvinylidene fluoride membrane; pore size: 30 nm) are used to retain the biomass, leading to a considerably high total suspended solid concentration. Finally, the filtrates collected from MBR are subjected to the DTRO process (cross-flow filtration; membrane type: polyamide composite membrane; salt rejection rate: > 98%) for further purification.

### 2.3. Fish exposure and sampling protocols

Goldfish are widespread worldwide; they are vulnerable to

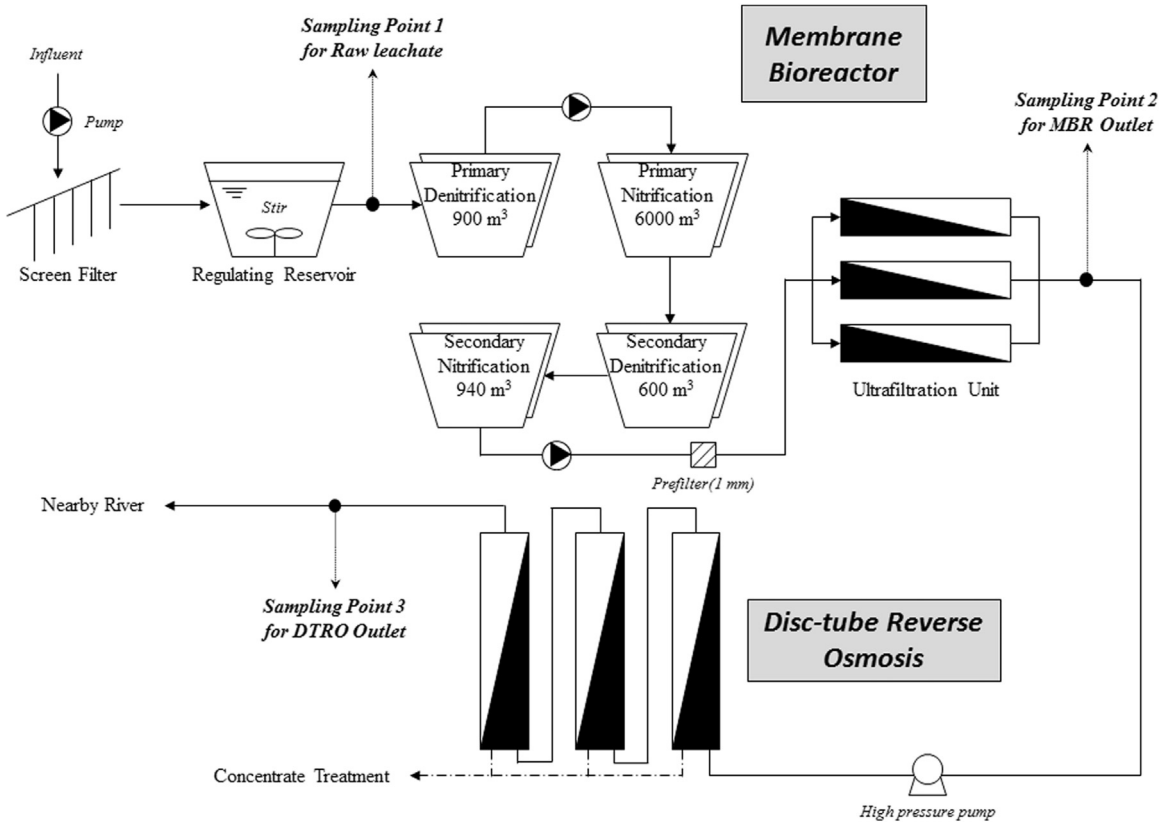


**Fig. 1.** Schematic drawing of the treatment process for the leachate with the sampling points (dots).