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# Effects of bacterial activity on estrogen removal in nitrifying activated sludge

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ARTICLE INFO

*Article history:*
Received 13 February 2007
Received in revised form
22 March 2007
Accepted 26 April 2007
Available online 8 May 2007

*Keywords:*
Estrogen
Nitrifying activated sludge
Bacterial activity
Co-metabolism

ABSTRACT

The effects of bacterial activity on the degradation of estrone (E1), estradiol (E2), estriol (E3) and ethinylestradiol (EE2) in nitrifying activated sludge (NAS) were studied with different substrates and organic loading rates (OLRs) and low temperature conditions. Heterotroph was shown to have utilized glucose prior to E1 for metabolism. The co-metabolism of ammonia oxidizing bacteria (AOB) dominated the degradation of E1, E2 and EE2 in NAS. The higher the organic loading, the higher the rate of organic matter transformation, with less ammonia oxidation and less degradation of E1, E2 and EE2. The degradation of E3 in NAS was shown to be largely due to heterotroph metabolism. On the basis of the difference of apparent activity between heterotroph and AOB at 4 °C, the process of estrogen degradation via the co-metabolism of AOB was able to be identified.



## 1. Introduction

Techniques in biological wastewater treatment range from the control of organic matter and suspended solids (SS), to the control of nutrients, and in recent years, also include the control of specific or trace contaminants. Although the amount of estrogens detected in treated wastewater is at nanogram levels or even lower, many studies have revealed that the presence of estrogens in treated wastewater is responsible for the feminization of male fish and sexual disruption in many aquatic wildlife (JEA, 1998; IUPAC, 2003). It has been reported that estrogens are efficiently removed in some sewage treatment plants (STPs) which use an activated sludge process. However, there are tremendous differences in the degree of estrogen removal among them, and in some cases, an increase in estrogenic activity or steroid estrogen concentration in the effluent and/or excess sludge has been shown, compared with that of the influent (Carballa et al., 2004; Matsui et al., 2000).

It is known that estrogens are either removed by direct use as electron donors for heterotrophs or via the co-metabolic degradation of ammonia oxidizing bacterium (AOB). Yoshimoto et al. (2004) have found that *Rhodococcus zopfii* and *R. equi* isolates from activated sludge can degrade estradiol (E2), estrone (E1) and estriol (E3). This is also true of ethinylestradiol (EE2) at an initial concentration of 100 mg/L. Shi et al. (2004) has showed that *Nitrosomonas europaea* is capable of oxidizing E2, E1, E3 and EE2 at 200 μg/L of estrogen added in the presence of ammonia.

Low efficiencies of estrogen removal are exhibited in most conventional activated sludge systems which operate with relatively short sludge retention times (SRTs) and do not include the implementation of a biological nutrient removal process (Carballa et al., 2004; Clara et al., 2005). The positive influence of long SRTs on nitrification in activated sludge systems has been also associated with increased estrogen removal. Kreuzinger et al. (2004) have observed that with the increase of the SRT, the biodegradation of estrogen also increased. Clara et al. (2005) showed that STPs with nitrogen removal also efficiently removed degradable micropollutants (including estrogen). On the other hand, it has been discovered that chemicals present in low concentrations (μg/L

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0043-1354/$ - see front matter © 2007 Published by Elsevier Ltd.
doi:10.1016/j.watres.2007.04.028

range or lower) may show quite different biodegradation behavior than they typically do at high concentrations (mg/L range); moreover, it appears that biodegradation often is favored at low concentrations, and in such cases, co-metabolic degradation mechanisms may dominate (Alexander, 1985). Thus, the usual parameters utilized to control the nitrification may also influence estrogen removal. Among these parameters, beside the SRT, the organic loading rate (OLR) is usually taken into account since the OLR of STPs with nutrient removal is far lower than that in conventional STPs. A study on detergent chemicals in trace concentration range has revealed that their removal was more complete in low loaded plants than in high loaded plants, and it has been generally believed that nitrifying plants show better removal efficiency than non-nitrifying plant (Giger 1987). Since AOB is generally inactive at high OLR, OLR is likely a key parameter that will influence estrogen removal via the co-metabolism of AOB in activated sludge system.

On the other hand, in our previous work, we observed that E2 was degraded and converted into E1 at 4 °C in nitrifying activated sludge (NAS) (Ren et al., 2007). Under this condition, heterotrophs showed no significant activity, but a stable nitrification was obtained. As a result, the degradation of E2 at 4 °C was considered to be due to the co-metabolism of AOB. However, further investigation is required to identify the different behaviors of heterotroph and AOB when estrogen is degraded at low temperatures.

The activity of heterotroph or AOB dominates the efficiency of the oxidation of organic matter or ammonia in activated sludge systems; however, it remains unclear whether the activities of these bacteria are also associated with the removal of estrogen at trace concentration levels. In this paper, three different approaches for controlling the activities of bacteria in activated sludge were carried out to study the effect of the activities of heterotroph and AOB on estrogen degradation. These approaches were: (1) different substrate feeding, (2) different OLRs and (3) a low temperature condition: 4 °C.

## 2. Materials and methods

### 2.1. Chemicals and reagents

The estrogens used were above 98% purity. The estrone, 17$\beta$-estradiol and estriol were purchased from Wako Pure Chemical Industries Ltd. (Osaka Japan). The 17$\alpha$-ethinylestradiol was purchased from Sigma-Aldrich Inc. (USA). All the organic solvents used were of HPLC grade. Stock solutions (1000 mg/L) of each estrogen were prepared in methanol in glass tubes and kept at −30 °C until use. The stock solutions were subject to ultrasonic treatment for 10 min, and then diluted to 50 or 5 mg/L working stock solutions, which were calibrated twice before being added using a high performance liquid chromatography–mass spectrometry 2010 (HPLC/MS) (Shimadzu, Tokyo).

### 2.2. Activated sludge for experiments

The activated sludge was obtained from a sequencing batch reactor with a volume of 20 L, acclimated an approximate one year feeding of a swine wastewater which was stored in polyethylene tanks at 5–8 °C until use. Estrogens could not be detected in the wastewater stored over three weeks or in the activated sludge for experimental use. The sludge was allowed to settle and the supernatant was decanted, and then the same volume of 0.1 M phosphate buffer was added to remove $NH_4^+$–N, $NO_2^-$–N, and $NO_3^-$–N contained in sludge and maintain the bacterial activity. This washing process was repeated three or four times until there were no significant changes in total organic carbon (TOC) and nitrogen. The determination of TOC and nitrogen was carried out by a TOC 5000 analyzer (Shimadzu Co.) and a Traacs 800 (Alfa-Laval, German), respectively. The washed sludge was then diluted with distilled water to a certain concentration for use.

### 2.3. Extraction and analysis of selected estrogen

Estrogens in the solution of NAS were extracted according to Japanese Standard Methods for Sewage Test (Japan Sewage Works Association. 2002) as described previously (Ren et al., 2007). For the detection of selected estrogen by HPLC/MS, a gradient elution from 30% to 90% acetonitrile in water in 20 min at a flow rate of 200 μL/min was used as mobile phase of HPLC. Separation was achieved on an Xterra © MS C18 column (3.5 μm, 2.1 × 100 mm. Waters) preceded by a guard column (Xterra © RP 18, 3.5 μm, Waters). MS detection was performed under the time-scheduled selected ion monitoring conditions by using an electrospray interface operating in the negative ion mode. MS conditions were as follows: nebulizing gas flow, 1.5 L/min; curved desolvation line (CDL) voltage, −25 V; CDL temperature, 250 °C; probe voltage, −4.5 V; and detector gain, 1.6 kV. Nitrogen was used as nebulizing and drying gas.

### 2.4. Estrogen biodegradation in NAS under different substrate conditions

Since the E1 was not only a primary product of E2 biotransformation in activated sludge, but also was detected in the effluent of STPs, the E1 was used as the typical estrogen in our study of estrogen biodegradation behavior in NAS under different substrate conditions. Synthetic wastewater, detailed in Table 1, was used to provide the trace nutrients for bacterial growth. Four different substrates, shown in Table 2, were prepared to study the estrogen removal behavior under different activities of heterotroph and AOB in NAS. The initial concentrations were 655 mg/L for glucose (as $COD_{Cr}$) and 54.7 mg/L for $NH_4Cl$ (as $NH_4$–N). The E1 was added at 300 μg/L in every flask. The pH was adjusted to approximately 7.7 by the addition of 40 g/L $Na_2CO_3$ and 0.5% HCl. Solutions of activated sludge (100 mL) containing 1420 mg/L of SS were added in 300 mL Erlenmeyer flasks. All flasks were agitated on an orbit shaker at 200 rpm in the dark in a 20 °C thermostatic room. The dissolved oxygen (DO) in the water was 8.2 mg/L at the beginning and 4.0 mg/L at the end. Glucose, $NH_4Cl$ and E1 were added again to the flasks after four days of shaking, with the total of eight days of agitation. Samplings were performed over this time with duplicates for the determination of E1, $COD_{Cr}$ and $NH_4$–N.