Microbial structure of nitrifying granules and their estrogens degradation properties

X. H. Wang and V. Ivanov

ABSTRACT

It has been known that endocrine disruption compounds such as natural estrogens estrone, 17β-estradiol, estriol, and synthetic steroid 17a-ethynylestradiol can be degraded by nitrifying bacteria. The aim of this research was to test biodegradation of estrogens by microbial granules containing nitrifying bacteria. Cultivation of microbial granules was performed in sequencing batch reactor in model wastewater with carbon to nitrogen ratio of 100:30 by weight. After the system reached the steady state, the mean diameter of granules, sludge volume index of granular biomass, and biomass concentration were 0.6 mm, 22 mL g^{-1} and 7 g L^{-1}, respectively. Nitrite was not detected in the effluent, and conversion of the consumed NH_4^+-N to NO_3^--N was 93%. Specific NO_3^--N production rate was 0.12 d^{-1} at the constant NH_4^+- loading late of 0.9 g L d^{-1}. The presence of ammonium-oxidizing and nitrite-oxidizing bacteria was confirmed by fluorescence in situ hybridization with 16S rRNA-targeted oligonucleotide probes Nsm156 and Nit3. The estrogen biodegradation by the granules was conducted in batch experiments. The complete biodegradation of the mixture of estrone, 17β-estradiol, estriol, and 17a-ethynylestradiol, 100 μg L^{-1} each, by microbial granules was within 60 days. The order of biodegradation for estrogens by microbial granules was as follows: 17β-estradiol - > estriol - > estrone - > 17a-ethynylestradiol. The degradation rate constants of estrogens by microbial granules were 0.19 d^{-1}, 1.26 d^{-1}, 0.14 d^{-1} and 0.11 d^{-1} for estrone, 17β-estradiol, estriol and 17a-ethynylestradiol, respectively.

Key words | biodegradation, estrogen, microbial granules, nitrification

X. H. Wang
CH2M Hill Singapore,
150 Beach Road,
34th Gateway West,
Singapore
E-mail: *xiaohui.wang@ch2m.com*

V. Ivanov
School of Civil and Environmental Engineering,
Nanyang Technological University,
50 Nanyang Avenue,
Singapore
E-mail: *cvivanov@ntu.edu.sg*

INTRODUCTION

Recently, the impact of endocrine disrupting compounds (EDCs) on potential alterations of humans and animals' endocrine systems cause scientific community and public concerns. Natural hormones such as estrone (E1), 17β-estradiol (E2), estriol (E3), and synthetic steroid 17a-ethynylestradiol (EE2), which is used for birth control and estrogen replacement therapy, belong to the most potent endocrine disrupters if discharged to environment (Purdom *et al.* 1994). The levels of estrogens in surface waters were found from 0 to 5 ng L^{-1} for E1, E3 and EE2, and 0 to 27 ng L^{-1} for E2 (Ying *et al.* 2002). The presence of these EDCs in surface waters and sediments has been primarily attributed to their incomplete removal in the sewage-treatment process (Gomes *et al.* 2003). According to Baronti *et al.* (2000), the removal rates of E1, E2, E3, and EE2 from wastewater in activated sludge treatment were 61, 87, 95 and 85%, respectively. The concentration of estrogenic steroids detected in effluents of wastewater treatment plants (WWTPs) of different countries was up to 70 ng L^{-1} for E1, 64 ng L^{-1} for E2, 18 ng L^{-1} for E3 and 42 ng L^{-1} for EE2 (Ying *et al.* 2002). At the downstream of certain WWTP effluent discharges in British rivers, the

doi: 10.2166/wst.2009.218

concentration of natural steroidal estrogens was sufficient to affect the level of vitellogenin synthesis in male fish (Routledge *et al.* 1998).

For complete removal of estrogens from wastewater, a variety processes such as membrane nanofiltration and extended activated sludge process (Gomes *et al.* 2003; Khan *et al.* 2004; Weber *et al.* 2004) were studied. Municipal WWTP with an activated sludge system for nitrification and denitrification could eliminate natural and synthetic estrogens (Ying *et al.* 2002; Andersen *et al.* 2003; Braga *et al.* 2005). Such phenomenon could be attributed to estrogen biodegradation properties of ammonia-oxidizing bacteria (Shi *et al.* 2004).

Microbial granules, used in wastewater treatment, have compact and strong structure, which in turn result in a better settling property, higher biomass retention and ability to withstand organic loading rate up to 15 g COD L^{-1} d^{-1} (Tay *et al.* 2001; Moy *et al.* 2002). These properties of granules ensure a possibility to create compact and cost-effective bioreactor for wastewater treatment when nutrient removal is a prerequisite (Yang *et al.* 2003; de Kreuk *et al.* 2004). Microbial granules have diverse microbial community, complex spatial structure, coordinated physiological functions and specific temporal changes (Tay *et al.* 2002; Ivanov *et al.* 2005). Microbial granules, contain nitrifying bacteria (Ivanov *et al.* 2005), are more effective in nitrification than activated sludge because of protection of sensitive nitrifiers from toxic substances and shock loading. Probably, nitrification and denitrification processes can be achieved simultaneously in microbial granules because the heterotrophic and nitrifying populations co-exist in one microbial granule (Tay *et al.* 2002; Liu *et al.* 2004).

The objective of this study was investigation of estrogens biodegradation by microbial granules containing nitrifying bacteria.

METHODS

Formation of microbial granules consisting heterotrophs and nitrifying bacteria

One column (120 cm in height and 5 cm in diameter) with working volume of 2.5 L was used as sequencing batch reactor (SBR) for formation of nitrifying granules, which was operated sequentially with a cycle time of 4 h (5 min of influent filling, 200–220 min of aeration, 10–30 min of settling and 5 min of effluent withdrawal). Effluent was discharged from the middle port of the reactor at a volumetric exchange of 50%. Fine air bubbles for aeration were introduced by a dispenser at the reactor bottom at an airflow rate of 2.5 L min^{-1}. The column reactor was housed in a temperature-controlled room at 25°C and started up by inoculating 1,200 mL of raw activated sludge taken from a municipal wastewater treatment plant in Singapore. Synthetic wastewater composed of 1,000 mg L^{-1} ethanol-COD, 300 mg L^{-1} ammonium nitrogen, 2,400 mg L^{-1} bicarbonate, trace elements and micronutrients (Moy *et al.* 2002).

Determination of process parameters

Ammonium nitrogen (NH_4^+—N) and COD were analyzed by standard method (APHA 1998). Nitrite nitrogen (NO^{3-}—N) and nitrate nitrogen (NO_3^-—N) were measured by flow injection analyzer (QuikChem Method 10-107-06-1-I, Lachat instruments Inc.). pH was measured by pH probe. Chemical oxygen demand (COD), mixed liquor suspended solids (MLSS), sludge volume index (SVI) were determined by standard methods (APHA 1998).

Preparation of granules for fluorescence in situ hybridization (FISH)

The granules, collected from the reactor, were fixed in a 3:1 ratio with 4% formaldehyde for 3 hours as described by Amann *et al.* (1995). Then, the samples were used for sectioning and hybridization. Granules were rinsed with 1 × phosphate buffered saline (PBS) and then embedded in Tissue Freezing Medium (Leica Instrument, GmbH, Germany) to freeze overnight at −20°C. Frozen granules were sectioned to a thickness of 50 μm using LEICA CM3050S-Cryostat at −20°C. Each granule section was placed on a gelatin coated slide glass. The section were dehydrated by successive passages through 50%, 80% and 98% (v/v) ethanol solutions and then air-dried before being used for fluorescent in situ hybridization (FISH).

Oligonucleotide probes and FISH

The following 16S rRNA-targeted oligonucleotide probes were used: (1) Nsm156 probe labeled with tetrachlorofluorescein (TET), (2) Nit3 probe labeled with the fluorochrome CY3, (3) Par651 probe labeled with fluorochrome CY5. The final probe concentration was approximately 5 ng μL^{-1}. The probe and hybridization conditions were listed in Table 1. The hybridization buffers contained 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% sodium dodecyl sulfate (SDS), and different concentration of formamide as listed in Table 4.1. Hybridization was performed for 3 h at 48°C in an isotonically equilibrated humid chamber; followed by a 30-min washing at 46°C with buffer containing 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% sodium dodecyl sulfate (SDS). For combinations of probes with different optimal hybridization stringencies, two hybridizations were done successively. The first hybridization was done with the probe which required the higher formamide concentration, and was followed by a second hybridization at a lower stringency.

The granule images were acquired by a Fluoview300 confocal laser scanning microscope (CLSM) (Olympus, Japan). Green and red fluorescence were excited by a 10 mW argon laser at 488 nm, separated with 570 nm splitting filter and detected in channel 1 with long pass filter 510 nm and in channel 2 with a band pass filter excited by a helium neon laser at 633 nm and measured in channel 2 with a long pass filter 660 nm.

Estrogen chemicals

The chemicals used in this study were estrone, 17β-estradiol, estriol, 17a-ethynylestradiol, and estrone-d4 (Sigma-Aldrich, St. Louis, MO, USA). Solution of 1 mg L^{-1} of estrogens was prepared by dilution of 10 μl of standard solution 1,000 mg L^{-1} of E1, E2, E3 and EE2 to 10 ml with methanol. Solution of estrone-d4 (E1-d4), 10 μg L^{-1}, was used as internal standard for E1, E2, E3 and EE2 measurements.

Estrogen biodegradation in batch experiments

An aliquote of 2 ml of activated sludge (mixed liquor suspended solids concentration was 3.0 g L^{-1}), diluted in 30 mL of minimal medium (K_2HPO_4, 1.0 g L^{-1}, $(NH_4)_2SO_4$, 1.0 g L^{-1}, NaCl, 0.1 g L^{-1}, $MgSO_4.7H_2O$, 0.2 g L^{-1}, $FeCl_3$, 0.01 g L^{-1}, $CaCl_2$, 0.1 g L^{-1}), was spiked with solution of mixed estrogens to obtain a concentration of 100 μg L^{-1} for each estrogen. Around 150 mg of wet microbial granules consisting nitrifying bacteria was inoculated in 30 mL of minimal medium with concentration of 100 μg L^{-1} of each estrogen. In order to maintain a homogenous suspension and aerobic conditions, the batch cultivation was in horizontal shaker at 150 rpm. At appropriate time intervals, 0.5 mL aliquots of the homogenized suspension were taken for liquid phase extraction and analysis. All batch experiments were performed in duplicates.

Estrogens in liquid

A sample of aliquot of 0.5 mL was transferred to 2 mL centrifuge tubes. Then, estrogens were extracted with 0.5 mL dichloromethane during vortex for 3 min. After extraction, the tube was centrifuged at 10,000 rpm for 3 min. Top layer of dichloromethane solution was then removed and vacuum dried for 15 min to dryness, then reconstituted with 0.5 mL acetonitrile/water (1:1) containing 10 μg L^{-1} of E1-d4 as internal standard. The tube was vortexed for 1 min and centrifuged for 3 min at 1,000 rpm. After that, the sample was transferred to HPLC vial for analysis with liquid chromatography/tandem mass spectrometry (LCMS/MS), which is composed as a high performance liquid chromatography HPLC 2010

Table 1 | 16S rRNA-targeted oligonucleotide probes used in this study

Probe	Sequence (5′-3′)	Binding position	Specificity	Formamide concentration (%, v/v)	Reference
Nsm156	TATTAGCACATCTTTCGTTCGAT	16S, 651-668	*Nitrosomonas cluster*	5	Juretschko *et al.* (1998)
Nit3	CCTGTGCTCCATGCTCCG	16S, 156-174	*Nitrobacter spp.*	40	Juretschko *et al.* (1998)

(Shimadzu, Tokyo, Japan) coupled to API 3000 triple quadrupole mass spectrometry (Applied Biosystems, Foster City, CA, USA). The 6-point calibration curve was performed with the respective estrogens at concentrations between 1 and 100 $\mu g\,L^{-1}$.

Estrogens in sludge

The method, which was used for measuring content of estrogens in granular sludge after 60 days of batch cultivation, was described by Andersen *et al.* (2003). Granules were freeze-dried, and then estrogens were successively extracted twice with methanol and subsequently twice with dichloromethane. For each extraction step, the slurry of the sample in solvent was ultrasonicated for 10 min. The four solvent fractions were combined, and centrifuged at 10,000 rpm for 3 min. The solvent was vacuum dried for 15 min to dryness, then reconstituted with 0.5 mL acetonitrile/water (1:1) containing 10 $\mu g\,L^{-1}$ of E1-d4 as internal standard, followed by 1 min vortex and 3 min centrifugation at 1,000 rpm. After that, the sample was transferred to HPLC vial for analysis with LCMS/MS.

RESULTS

Formation of granules for nitrification in SBR

Figure 1 shows the time course of process in SBR. After 17 days of cultivation, there was no nitrification. At day 17, the reactor was inoculated with 100 mL of nitrifying enrichment culture with MLSS of 1.5 $g\,L^{-1}$. From day 18, nitrification started up. Granules with nitrification ability were formed during next 3 weeks (Figure 2). The system reached the steady state in terms of COD removal efficiency and complete nitrification in each cycle after 40 days. The mean diameter of granules, sludge volume index of granular biomass, and biomass concentration in the samples collected on day 55 were 0.6 mm, 22 $mL\,g^{-1}$ and 7 $g\,L^{-1}$, respectively. Nitrite was not detected in the effluent and ammonium consumption efficiency was close to 100%. Conversion of NH_4^+-N to NO_3^--N was 93% and the specific NO_3^--N production rate was 0.12 d^{-1} at the constant NH_4^+-N loading rate of 0.9 $g\,L^{-1}\,d^{-1}$. Figure 2 shows the

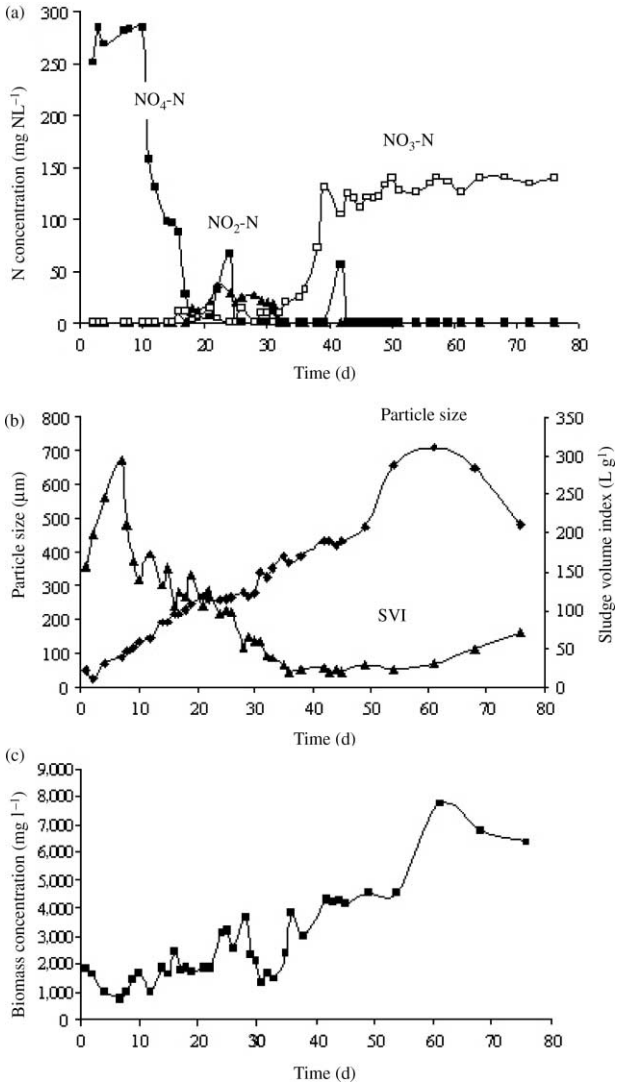

Figure 1 | Time course of parameters in process of SBR operation.

time course of parameters in one cycle of SBR. Complete nitrification was finished during 3 h. pH in one cycle first decreased from 7.8 to 7.1, and then reached to a steady state.

Detection of AOB and NOB in granules

To investigate the spatial distributions of AOB, and NOB within granules, fluorescence in situ hybridization with specific probes Nsm156 and Nit3 was carried. Both ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) were detected mainly in the layer of 50 to 200 μm from the surface of granule.

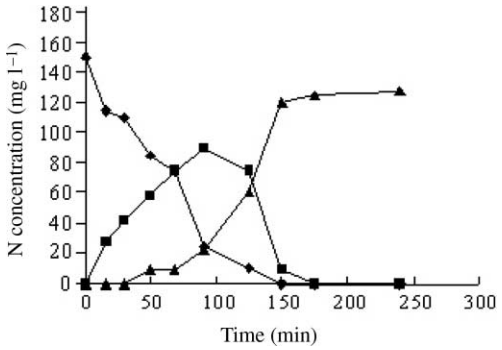

Figure 2 | Time course of parameters in one cycle of SBR operation. ◆: NH_4^+-N concentration; ■: NO_2^--N concentration; ▲: NO_3^--N concentration.

Estrogens biodegradation test in batch experiments

Using activated sludge for biodegradation test, E2 concentration dropped from 110 ± 14 to 0 $\mu g\,L^{-1}$ within 10 days, E3 concentration remained stable at around 100 $\mu g\,L^{-1}$, and E2 concentration dropped below detection limit. E1 concentration was gradually increased from 26 ± 3 $\mu g\,L^{-1}$ to 123 ± 12 $\mu g\,L^{-1}$ on day 8, and remained at this level till day 10. E1 was degradated very fast. The concentration of E1 was dropped from 120 ± 1 at day 10 to 9 μg ± 3 $\mu g\,L^{-1}$ at day 12, while there was no further decrease of E1 concentration since day 12. EE2 concentration was stable at around 122 $\mu g\,L^{-1}$, as showed in Figure 4.

Using microbial granules containing AOB and NOB for biodegradation test, all four estrogens were degraded (Figure 4). E2 was totally removed from liquid medium in one day, E3 concentration decreased from 121 ± 0.5 $\mu g\,L^{-1}$ at day 1 to 10 ± 1 $\mu g\,L^{-1}$ at day 21. E1 concentration was increased from 20 ± 3 $\mu g\,L^{-1}$ to 150 ± 20 $\mu g\,L^{-1}$ in day 1 and then remained at this level till day 12, followed by a decreasing. The complete biodegradation of E1 was achieved by day 30. Followed by E1 degradation, there was EE2 biodegradation by microbial granules containing nitrifying bacteria. EE2 concentration dropped from 115 $\mu g\,L^{-1}$ to 3 ± 0.5 $\mu g\,L^{-1}$ at day 60. The estrogens degradation rate constants were 0.19 d^{-1}, 1.26 d^{-1}, 0.14 d^{-1} and 0.11 d^{-1} for E1, E2, E3 and EE2, respectively.

The measured content of E1 and EE2 in microbial granules after 60 days were 407 and 83 $ng\,g^{-1}$ granules (dry), respectively. The contents of E2 and E3 in granules were below detection limit.

DISCUSSION

Chemilithoautotrophic nitrifying bacteria play an important role in natural cycling of nitrogen. They are used widely in wastewater treatment system for nitrogen removal using aerobic nitrification followed by denitrification. The sequential transformation of ammonium to nitrate via nitrite is performed by two groups of bacteria, autotrophic AOB and NOB. In SBR of this research, 150 $mg\,L^{-1}$ of ammonium was completely converted to nitrate via nitrite within the first 3 h of cycle. Considering that COD of cycle was 500 $mg\,L^{-1}$ and assuming ammonium assimilation rate by heterotrophic bacteria was 20% of COD loading (Yang *et al.* 2004), the assimilated NH_4^+-N by heterotrophic bacteria is 100 $mg\,L^{-1}$. Given 150 $mg\,L^{-1}$ NH_4^+-N concentration at the beginning of each cycle, it can be assumed that autotrophic nitrifying bacteria present in cultivated microbial granular sludge. The presence of AOB and NOB in aerobically grown microbial granules was further confirmed by FISH. Oligonucleotide hybridization probe Nsm156 and Nit3 were used to detect AOB and NOB. Nit3 probe, which is specific for *Nitrobacter* spp., was selected because ammonium concentration in this study was higher than 100 mg-N L^{-1}. It has been reported that the presence of *Nitrospira*-like bacteria, another nitrite-oxidizing bacteria were frequently found only in nitrification reactor with concentration of ammonium lower than 100 mg-N L^{-1} (Tsuneda *et al.* 2003). Nevertheless, the total number of NOB was extremely small compared with that of AOB (Figure 3b). This was probably because the nitrate production rate of *Nitrobacter* sp. (5.1–42 fmol $cell^{-1}\,1\,h^{-1}$) was superior to the nitrite production rate of *Nitrosomonas* sp. (0.9–2.0 fmol $cell^{-1}\,h^{-1}$) (Prosser 1989).

The research on the role of nitrifying bacteria in estrogens biodegradation is very limited. However, Shi *et al.* (2004) showed that both natural estrogens E1, E2 and E3 and synthetic estrogen EE2 can be biodegraded by *Nitrosomonas europaea*, which was also able to oxidize hydrocarbons and their derivatives, such as methane, methanol, phenol and benzene, and halogenated hydrocarbons (Hyman *et al.* 1988). In this study, E1 was generated when microbial granules degraded E2 (Figure 4b), whereas there was no formation of E1 observed when *N. europaea*

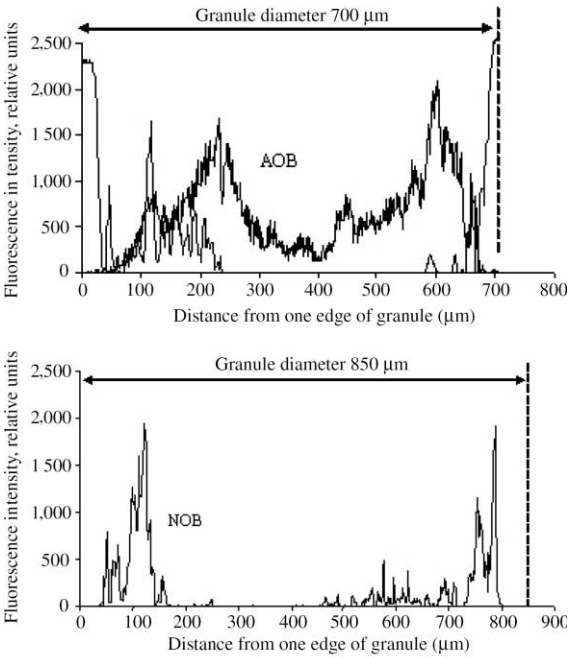

Figure 3 | Distribution of AOB and NOB within a cross-section of different size-categorized granules from an aerobic granular sludge sequencing batch reactor.

degraded E2 (Shi *et al.* 2004). Therefore, *N. europaea*, and the microbial granules containing nitrifying bacteria, exhibit different E2 biodegradation pathways. As E1 accumulation was observed during E2 biodegradation by conventional activated sludge, E2 biodegradation via E1 could be caused by other heterotrophic bacteria or other than *N. europaea*. nitrifying bacteria in microbial granules. By microbial granules containing nitrifying bacteria, estrogens degradation constant rates were $0.19\,d^{-1}$ for E1, $1.26\,d^{-1}$ for E2, $0.14\,d^{-1}$ for E3 and $0.11d^{-1}$ for EE2. These values are similar to the estrogens biodegradation rate of nitrifying activated sludge (Shi *et al.* 2004).

Estrogens degrader or nitrifying activated sludge was tested with single estrogen. For example, studies on degradation of estrogens by nitrifying activated sludge showed the degradation rate obeyed the first order reaction kinetics with degradation rate constants of $31.2\,d^{-1}$ for E2, $0.72\,d^{-1}$ for E3, $1.34\,d^{-1}$ for E1, and $0.84\,d^{-1}$ for EE2 (Shi *et al.* 2004), indicating E2 was most fast biodegraded among these four estrogens. However, the natural and synthetic estrogens in both natural water and wastewater are generally present simultaneously. The biodegradation pattern for mixed estrogens could significantly affect their

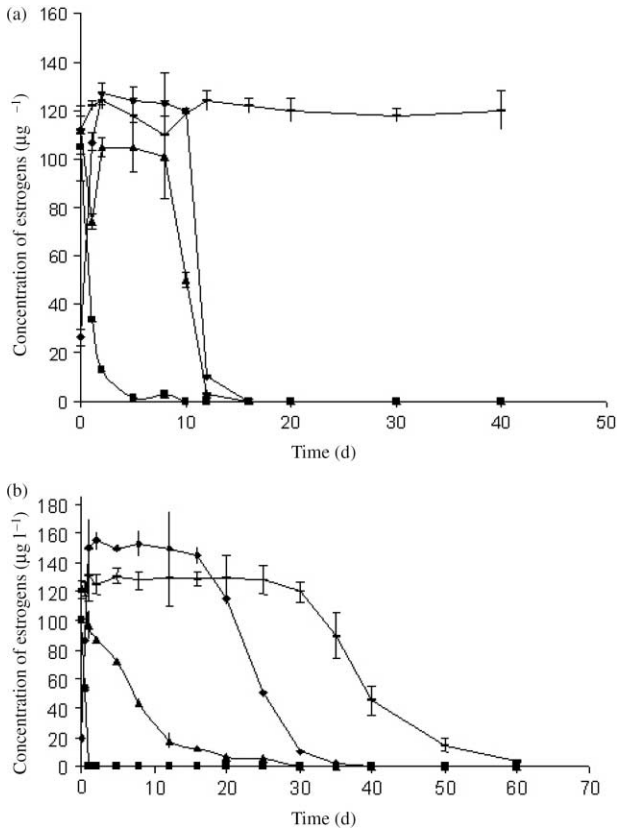

Figure 4 | Biodegradation of estrogens by activated sludge and microbial granules containing nitrifying bacteria AOB and NOB. (A). Treatment with activated sludge; (B) Treatment with microbial granules containing nitrifying bacteria AOB and NOB. ■: E2, ▲: E3, ◆: E1, —: EE2.

environmental fate and retention time of each estrogen in water. The mixed estrogens biodegradation patterns, such as simultaneously or sequential, could provide valuable reference on design parameters such as solid retention time and hydraulic retention time in biological wastewater treatment process when estrogens removal is a prerequisite. To get such information, each of $100\,\mu g\,L^{-1}$ solution of E1, E2, E3 and EE2 were pre-mixed for estrogens biodegradation test. Estrogens biodegradation follows the sequence of E2>E3>E1>EE2 by both activated sludge and microbial granules containing nitrifying bacteria.

It has been reported that natural estrogens E2 and E3 could relatively easier be removed in biological wastewater treatment. Whereas, the presence of E1 and synthetic estrogen EE2 dominated in WWPT effluent (Ternes & Kreckel 1999; Andersen *et al.* 2003; Joss *et al.* 2004; Braga *et al.* 2005). Such relatively high stability of E1 and EE2 in wastewater treatment could be due to their lower

biodegradatability. Therefore, short hydraulic retention time (HRT) or low sludge retention time (SRT) of the conventional continuous biological wastewater treatment process will result in incomplete biodegradation of E1 and EE2. To complete removal of these estrogens, sufficient HRT and SRT are necessary. For example, EE2 was degraded significantly (about 90%) only when the Wiesbaden plant was upgraded to remove nutrients with SRT = 11–13 d from previous SRT < 4 d (Andersen *et al.* 2003). This enhancement could be due to the growth of microorganisms such as nitrifying bacteria, capable of degrading EE2 in upgraded nutrient-removal plant. In the current study, EE2 was completely removed from liquid solution by microbial granules containing nitrifying bacteria, whereas there was no reduction of EE2 during the batch experiments with conventional activated sludge.

The reduction of estrogens in liquid medium by microbial granules could be either by biodegradation or bio-sorption of estrogens onto granular sludge, or both. Given the relatively low polarity of these compounds, which octanol-water partition coefficients were mostly between 2.5 and 5, estrogens could be removed by sorption on primary and secondary sludge (Petrovic *et al.* 2001). Nevertheless, it was identified that adsorption of estrogens by activated sludge and river sediments was as low as 11.9 and 22.8 $ng\,g^{-1}$ for E1 and EE2 respectively. The hormone fraction found in suspended matter in WWPT represented less than 10% of total concentration (EE2 + E2 + E1) of both influent or effluent (Lopez de Alda 2001; Ternes *et al.* 2002; Andersen *et al.* 2003). In this study, the adsorption of E1 and EE2 by microbial granules was 407 $ng\,g^{-1}$ and 83 $ng\,g^{-1}$, respectively. This showed that microbial granules have higher estrogen adsorption capacity than that of activated sludge. By mass balance calculation, the estrogens fraction, found in microbial granules, represented less than 0.5% of total concentration (E1 + EE2) in batch experiments. Therefore, it can be concluded that the biodegradation was major process in estrogens reduction during batch experiments with microbial granules containing nitrifying bacteria.

Nitrifying activated sludge can diminish concentrations of both natural and synthetic estrogens (Shi *et al.* 2004). However, nitrification is generally a limiting step for nutrient removal in conventional activated sludge system, because AOB and NOB are belonging to autotrophic microorganisms, which are extremely sensitive to the environmental conditions like temperature, pH, light, toxic substance, and shock loading. Granular biomass system is more robust than conventional activated sludge system. It has higher resistance for toxic substances and can more rapidly recover after physiological shock. As investigated by Liu *et al.* (2005), for initial phenol concentration of 20 $mg\,L^{-1}$, the recovery of nitrification was 67% of the control, which was activated sludge. It shows that nitrifying bacteria, embedded in microbial granules, are relatively resilient to the inhibitory effects exerted by phenol. Soljan-Glancer *et al.* (2001) also showed that granulated biomass, containing nitrifying bacteria, can retain in bioreactor for a longer time than in conventional activated sludge. This study is first time demonstration of the applicability of nitrifying microbial granules for removal of estrogens from wastewater.

CONCLUSIONS

Aerobically grown microbial granules, which containing nitrifying bacteria AOB and NOB, were cultivated in SBR with C/N of 1:0.3.

The presence of ammonium-oxidizing and nitrite-oxidizing bacteria was confirmed by fluorescence in situ hybridization with 16S rRNA-targeted oligonucleotide probes Nsm156 and Nit3.

The order of biodegradation for estrogens by microbial granules was as follows: 17β-estradiol > estriol > estrone > 17a-ethynylestradiol.

The complete biodegradation of the mixture of estrone, 17β-estradiol, estriol, and 17a-ethynylestradiol, 100 $\mu g\,L^{-1}$ each, by microbial granules was within 60 days.

The degradation rate constants of estrogens by microbial granules were 0.19 d^{-1}, 1.26 d^{-1}, 0.14 d^{-1} and 0.11 d^{-1} for estrone, 17β-estradiol, estriol and 17a-ethynylestradiol, respectively.

REFERENCES

Amann, R. I., Ludwig, W. & Schleifer, K.-H. 1995 Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**(1), 143–169.

Andersen, H., Siegrist, H., Halling-Sorensen, B. & Ternes, T. A. 2003 Fate of estrogens in a municipal sewage treatment plant. *Environ. Sci. Technol.* **37**, 4021–4026.

APHA 1998 *Standard Methods for the Examination of Water and Wastewater*, 19th edition. American Public Health Association, Washington, DC, USA.

Baronti, C., Curini, R., D'Ascenzo, G., Di Corcia, A., Gentili, A. & Samperi, R. 2000 Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environmental Science and Technology* **34**(24), 5059–5066.

Braga, O., Smythe, G. A., Schafer, A. I. & Feitz, A. J. 2005 Fate of steroid estrogens in australian inland and coastal wastewater treatment plants. *Environ. Sci. Technol.* **39**, 3351–3358.

de Kreuk, M., de Bruin, L. M. M. & Van Loosdrecht, M. C. M. 2004 Aerobic granular sludge-from idear to pilot plant. In: Bathe, S., de Kreuk, M., McSwain, B. & Schwarzenbeck, N. (eds) *Aerobic Granular Sludge*. IWA Publishing, London, pp. 111–124.

Gomes, R. L., Scrimshaw, M. D. & Lester, J. N. 2003 Determination of endocrine disrupters in sewage treatment and receiving waters. *Trends Anal. Chem.* **22**, 697–707.

Hyman, M. R., Murton, I. B. & Arp, D. J. 1988 Interaction of ammoniua monooxygenase from nitrosomonas europea with alkanes, alkenes and alkynes. *Appl. Environ. Microbiol.* **54**, 3187–3190.

Ivanov, V., Tay, J.-H., Liu, Q.-S., Wang, X.-H., Wang, Z.-W., Maszenan, B. A. M., Yi, S., Zhuang, W.-Q., Liu, Y.-Q., Pan, S. & Tay, S. T.-L. 2005 Microstructural optimization of wastewater treatment by aerobic granular sludge. In: Bathe, S., Kreuk, Md., McSwain, B. & Schwarzenbeck, N. (eds) *Aerobic Granular Sludge*. IWA Publishing, London, pp. 43–52.

Joss, A., Andersen, H., Ternes, T., Richle, P. R. & Siegrist, H. 2004 Removal of estrogens in municipal wastewater treatment under aerobic and anaerobic conditions: consequences for plant optimization. *Environ. Sci. Technol.* **38**, 3047–3055.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.-H., Pommerening-Röser, A., Koops, H.-P. & Wagner, M. 1998 Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations. *Applied and Environmental Microbiology* **64**, 3042–3051.

Khan, S. J., Wintgens, T., Sherman, P. J. Z. & Schäfer, A. I. 2004 Removal of hormones and pharmaceuticals in the advanced water recycling demonstration plant in Queensland, Australia. *Water Sci. Technol.* **50**(5), 15–22.

Liu, Y., Yang, S.-F. & Tay, J.-H. 2004 Improved stability of aerobic granules by selecting slow-growing nitrifying bacteria. *J. Biotechnol.* **108**, 161–169.

Liu, Y.-Q., Tay, J.-H., Ivanov, V., Moy, B. Y.-P., Yu, L. & Tay, S. T.-L. 2005 Influence of phenol on nitrification by microbial granules. *Process Biochem.* **40**, 3285–3289.

Lopez de Alda, M. J. & Barcelo, D. 2001 Use of solid-phase extraction in various of its modalities for sample preparation of estrogens and progestogens in sediment and water. *J. Chromat. A* **938**, 145–153.

Moy, B. Y. P., Tay, J. H., Toh, S. K., Liu, Y. & Tay, S. T. L. 2002 High organic loading influences the physical characteristics of aerobic granules. *Lett. Appl. Microbiol.* **34**, 407–412.

Petrovic, M., Eljarrat, E., Lopez de Alda, M. J. & Barcelo, D. 2001 Analysis and environmental levels of endocrine-disrupting compounds in freshwater sediments. *Trends Anal. Chem.* **20**, 637–648.

Prosser, J. I. 1989 Autotrophic nitrification in bacteria. *Adv. Microbial. Physiol.* **30**, 125–181.

Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R. & Sumpter, J. P. 1994 Estrogenic effects of effluents from sewage treatment works. *Chem. Ecol.*, 8.

Routledge, E. J., Sheahan, D., Desbrow, C., Brighty, G. C., Waldock, M. & Sumpter, J. P. 1998 Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. *Environ. Sci. Technol.* **32**, 1559–1565.

Shi, J., Fujisawa, S., Nakai, S. & Hosomi, M. 2004 Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium Nitrosomonas europaea. *Water Res.* **38**, 2323–2330.

Soljan-Glancer, M., Ban, S., Landeka Dragicevic, T., Soljan, V. & Matic, V. 2001 Granulated mixed microbial culture suggesting successful employment of bioaugmentation in the treatment of process wastewaters. *Chem. Biochem. Eng.* **15**, 87–94.

Tay, J.-H., Liu, Q.-S. & Liu, Y. 2001 Microscopic observation of aerobic granulation in sequential aerobic sludge blanket reactor. *J. Appl. Microbiol.* **90**, 1–8.

Tay, J.-H., Ivanov, V., Pan, S. & Tay, S. T.-L. 2002 Specific layers in aerobically grown microbial granules. *Lett. Appl. Microbiol.* **34**, 254–257.

Ternes, T. Kreckel, P. & Mueller, J. 1999 Behaviour and occurence of estrogens in municipal sewage treatment plants-II. Aerobic batch experiments with activated sludge. *Sci. Total Environ.* **225**, 91–99.

Ternes, T. A., Andersen, H., Gilberg, V. & Bonerz, M. 2002 Determination of estrogens in sludge and sediments by liquid extraction and GC/MS-MS. *Anal. Chem.* **74**, 3498–3504.

Tsuneda, S., Nagano, T., Hoshino, T., Ejiri, Y., Noda, N. & Hirata, A. 2003 Characterization of nitrifying granules produced in an aerobic upflow fluidized bed reactor. *Water Res.* **37**, 4965–4973.

Weber, S., Gallenkemper, M., Melin, T., Dott, W. & Hollender, J. 2004 Efficiency of nanofiltration for the elimination of steroids from water. *Water Sci. Technol.* **50**, 9–14.

Yang, Q., Li, X., Zeng, G., Xie, S. & Liu, J. 2003 Cultivation of aerobic granular sludge for simultaneous nitrification and denitrification in SBR system. *Environ. Sci.* **24**, 94–98.

Yang, S.-F., Tay, J.-H. & Liu, Y. 2004 Inhibition of free ammonia to the formation of aerobic granules. *Biochem. Eng.* **17**, 41–48.

Ying, G. G., Kookana, R. S. & Ru, Y. J. 2002 Occurrence and fate of hormone steroids in the environment. *Environ. Int.* **28**, 545–551.