Environmental Science
Water Research & Technology

PAPER

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Cite this: *Environ. Sci.: Water Res. Technol.*, 2017, **3**, 1051

Estrone biodegradation in laboratory-scale systems designed for total nitrogen removal from wastewater†

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Changes in regional regulations are causing a shift towards the implementation of total nitrogen removal technologies. Conventional nitrification systems do not remove total nitrogen, instead only oxidizing ammonia and ammonium in the influent to nitrate. Conventional nitrification does, however, result in degradation of estrone (E1), a major contributor to the estrogenicity of wastewater treatment plant (WWTP) effluent. The objective of this research was to provide guidance on the impact that changes in wastewater treatment practices could have on E1 degradation. This was accomplished by comparing E1 removal in a laboratory-scale conventional nitrification system with that in a range of idealized laboratory-scale systems designed to remove total nitrogen from wastewater: the modified Ludzack-Ettinger (MLE) system (a two-stage anaerobic–aerobic system with recycle), a granular activated sludge system (cycled anaerobic–aerobic), a sequencing batch reactor (cycled anaerobic–aerobic), and an anaerobic ammonia oxidation (anammox) system. As anticipated, E1 removal was excellent when fed to the nitrification, MLE, and sequencing batch reactors, at >96% mean E1 loss. The granular activated sludge system operated in our laboratory failed to remove E1, which was perhaps not unexpected given the high COD loading under which our system was operated. Despite the anaerobic nature of anammox, it also resulted in excellent E1 removal (95% mean E1 loss) without concomitant 17β-estradiol production. This work demonstrates that the choice of nitrogen removal technology used by a treatment plant could have an impact on the estrogenicity of WWTP effluent, but low energy total nitrogen removal systems do exist that are capable of excellent E1 removal.

Received 23rd May 2017,
Accepted 25th July 2017

DOI: 10.1039/c7ew00164a

rsc.li/es-water

Water impact

New rules regarding nitrogen levels in wastewater treatment plant (WWTP) effluents may result in widespread implementation of total nitrogen removal technologies. Conventional nitrification systems do not remove total nitrogen, instead only oxidizing ammonia and ammonium in the influent to nitrate. These systems do provide the additional benefit of degrading estrone (E1), a human hormone and major contributor to the estrogenicity of WWTP effluent, however. The objective of this research was to provide guidance on the impact that changes in wastewater treatment practices could have on E1 degradation. This was accomplished by comparing E1 removal in a laboratory-scale conventional nitrification system with that in a range of idealized laboratory-scale systems designed to remove total nitrogen from wastewater: the modified Ludzack-Ettinger (MLE) system (a two-stage anaerobic–aerobic system with recycle), a granular activated sludge system (cycled anaerobic–aerobic), a sequencing batch reactor (cycled anaerobic–aerobic), and an anaerobic ammonia oxidation (anammox) system. This work demonstrates that the choice of nitrogen removal technology used by a treatment plant could have an impact on the estrogenicity of WWTP effluent and that low energy total nitrogen removal systems do exist that are capable of excellent E1 removal.

Introduction

As we exceed planetary boundaries on nutrients, regional regulatory directives to remove total nitrogen from wastewater are likely to increase.[1–3] Traditionally, a combination of nitrification followed by denitrification has been used for total nitrogen removal from wastewater. This process is reliable, but is also energy- and in some cases, material-intensive. Energy costs are significant for an individual plant, typically accounting for 15–40% of a wastewater treatment plant's (WWTP's) budget.[4] Between 30 and 50% of a wastewater treatment

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† Electronic supplementary information (ESI) available: Further details on the experimental set-up, analytical procedures, and additional effluent E1 and N data for several of the experiments are presented in Supporting Information. See DOI: 10.1039/c7ew00164a

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plant's energy consumption comes from aeration during aerobic operation.[5,6] Pumping, as required in some nitrification–denitrification systems, also consumes a significant portion of energy, consuming from 10–15% of the total energy used at a plant.[5] Other denitrification systems rely on the addition of an external carbon source, which may represent a substantial operating cost. Finally, an additional resource constraint for many WWTPs is land availability; a plant without additional space for the expansion of treatment capacity must consider the footprint of any new technology to be added, including a denitrification step coupled to nitrification.

Fortunately, viable new processes that facilitate the transformation of influent ammonium (NH_4^+) to harmless dinitrogen gas (N_2) while minimizing energy use and/or chemical addition, and in some cases plant footprint, are increasing in number.[7] Anaerobic ammonia oxidation (anammox) and granular activated sludge are two promising emerging nitrogen removal technologies.[7] Anammox microorganisms anaerobically convert stoichiometric quantities of NH_4^+ and nitrite (NO_2^-) to N_2 in a single step without oxygen input.[8] Some oxygen is required to generate NO_2^-, but the aeration, and therefore energy requirements, are much lower than that of traditional nitrification.[9] Granular activated sludge systems utilize sequencing batch reactors (SBRs); this facilitates small wastewater treatment plant footprints through simultaneous COD removal, nitrification, and denitrification in one reactor, very long cell, or solids, residence times (SRT), and high biomass concentrations.[10,11] Microbial granules form with nitrifiers in contact with the bulk liquid on the outside of the granule, and denitrifiers shielded from dissolved oxygen on the inside of the granule.[12] A granular activated sludge system is operated with intermittent aeration, reducing energy costs, and a very short sedimentation period to select for large, fast-settling granules,[13] which also facilitates excellent settling. High chemical oxygen demand (COD) loading and high shear in the reactor are additional parameters that promote granule formation.[11]

Beyond predictability, one additional benefit of traditional nitrification and denitrification is the fact that estrogens, such as estrone (E1), can be effectively degraded concomitant with nitrification.[14,15] E1, a natural human estrogen excreted in urine, is one of the major estrogens present in wastewater effluent and is subject to variable removal.[16–20] E1 is biodegraded during aerobic wastewater treatment,[21–23] primarily by slow-growing heterotrophic organisms that aerobically degrade E1 while also degrading multiple low-concentration organic substrates.[14,24] As a result, E1 degradation is favored in the presence of low concentrations of microbiologically derived carbon,[24,25] long cell residence times,[14,23,26] and aeration.[22,23] Nevertheless, the range of conditions under which E1 degradation can occur is broader and has not been fully explored. Two pure cultures have been isolated that are capable of using E1 (1 mM) as their sole electron donor and nitrate as their electron acceptor,[27,28] and one study has also shown excellent E1 degradation (approximately 100%) in a one-stage nitritation/anammox process in the presence of low dissolved oxygen concentrations (0.6–1.2 mg L^{-1}).[29] Another recent study[30] demonstrated approximately 62% E1 loss as a result of both abiotic and biological degradation and sorption in an anammox batch reactor treating synthetic urine. Abiotic nitration of E1 has also been shown to occur in the presence of high concentrations of NO_2^-, but this is not a significant pathway for E1 removal under typical wastewater conditions.[31]

Given the potential energy and material savings and smaller footprints of alternative total nitrogen removal technologies, it is important to better understand how estrogens might degrade in these newer processes, so that process decisions can be made based on a more complete ecological risk analysis. It is not intuitive how the conditions under which anammox or granular activated sludge systems operate will impact E1 degradation, however.[14,24] Therefore, the objective of this research was to experimentally determine, in idealized systems at the laboratory scale, whether E1 was degraded during the steady state operation of a traditional nitrification–denitrification (modified Ludzack-Ettinger), anammox, and granular activated sludge system; a traditional nitrification system served as a positive control. This research should help plants simultaneously consider E1 removal and their chemical, energy, and physical footprint as they adapt to stricter nitrogen regulations.

Experimental section

Reactor seed

Each reactor experiment, excluding the anammox experiments, was seeded with a 10 mL aliquot of concentrated activated sludge collected once from the Metropolitan WWTP in St. Paul, MN. More information on the preparation of the aliquots is provided in the ESI† (section S1). Two anammox experiments were performed and both were seeded with 50% by volume sludge taken from a full-scale DEMON System (York River WWTP, Seaside, VA) and stored at 4 °C until use. After an upset in the first anammox experiment (days 13–20), the reactor was reseeded with an additional 10% by volume DEMON sludge on day 20 of that experiment.

Overall reactor set-up and operation

Lab-scale nitrification and nitrogen removal experiments were performed using three unique reactor systems, described in detail in the ESI† (section S2). These systems were tested in five different experimental set-ups: traditional nitrification (duplicated); modified Ludzack-Ettinger (MLE), anammox (duplicated), granular activated sludge, and sequencing batch mode.

The influent composition, reactor volume, hydraulic retention time (HRT), SRT, and temperature used for each reactor set-up and experiment are given in Table 1. Peristaltic pumps were used to control the influent flow rate in all experiments. In every experiment the reactor influent solution was amended with 10 μg L^{-1} E1. To prevent the addition of solvent to the influent solution, the required volume of E1 in

Table 1 Reactor operation

Experiment	Reactor type	Influent composition	Reactor volume (L)	HRT (hours)	SRT (days)
Nitrification	CSTR[a]	Wastewater	0.8	5	10
MLE	CSTR	Wastewater	Anaerobic: 0.2 Aerobic: 0.8	10	10
Anammox	SBR[b]	Synthetic wastewater	Two experiments conducted: 1 and 0.25	12	Not controlled[c]
Granular activated sludge	SBR	Synthetic wastewater	2	12	Not controlled
Sequencing batch reactor	SBR	Synthetic wastewater	2	12	Not controlled

[a] Continuously stirred tank reactor. [b] Sequencing batch reactor. [c] Solids were not purposefully wasted from experiment.

methanol was added to empty plastic influent containers the day before the wastewater or synthetic wastewater was added, to allow the methanol to volatilize. In the case of the second (0.25 L) anammox experiment, the E1 was added to the influent container dissolved in water. All reactors were operated at a temperature of 21 ± 2 °C (average ± standard deviation, used throughout), except for the anammox reactors, which were operated at 30 °C. The nitrification and MLE reactors were continuously stirred.

Nitrification and modified Ludzack-Ettinger experiments

The nitrification experiments, performed in duplicate, utilized the schematic shown in Fig. SI-1.† The MLE experiment was performed in the reactor shown in the schematic in Fig. SI-2.† A membrane (Minikros® 750 kDa mPES cross flow filtration membranes, Spectrum Labs) was used as a clarifier in these experiments to separate biomass from the liquid, enabling the decoupling of the SRT and HRT. During use, membranes were backwashed daily, or when the pressure in the membrane feed lines exceeded 5 PSI.

Primary effluent was collected weekly at the Metropolitan WWTP and held at 4 °C until used. As stated above, E1 was amended to the wastewater prior to feeding the reactors. After E1 amendment, the COD, total nitrogen, and E1 in the influent to the nitrification and MLE reactors were measured to be 299 ± 80 mg L^{-1} (n = 8), 47 ± 78 mg L^{-1} (n = 16), and 6.2 ± 3.1 μg L^{-1} (n = 16), respectively, where n is the number of replicate samples analyzed. The influent flow rates were 2.67 mL min^{-1} and 1.67 mL min^{-1} for the nitrification and MLE experiments, respectively. Air was introduced to the aerobic reactors (see Fig. SI-1 and SI-2†) *via* a diffuser. The aerobic reactor in the nitrification system maintained a DO of greater than 5 mg L^{-1} throughout the duplicate experiments. The aerobic reactor in the MLE experiment was adjusted to maintain a DO of >2 mg L^{-1}. The anaerobic reactor in the MLE experiment received recycled oxygenated mixed liquor at two times the influent flow rate (2Q), and maintained a DO of less than 0.2 from day 0 to 13 and less than 0.4 from day 13 to 30. On day 30 and day 33 the DO in the anaerobic reactor increased to 0.5–0.7 mg L^{-1} then returned to ≤0.4 mg L^{-1} until the end of the experiment. To control the SRT, 80 and 100 mL of mixed liquor was removed daily from the nitrification and aerobic MLE reactors, respectively. The E1 concentration in the effluent was analyzed with time.

Anammox experiment

Two anammox experiments were conducted, nearly identical to each other with the exception of the reactor size, one conducted in a 1 L SBR and one conducted in a 0.25 L SBR. Experiments were conducted according to the schematic in Fig. SI-3† and the operational parameters in Table 1. The anammox feed, adapted from van de Graaf *et al.*[32] (Table SI-1†), was amended with 10 μg L^{-1} E1. After amendment, E1 was measured in the influent at 4.5 ± 0.6 μg L^{-1} (n = 3) in the 1 L reactor and 13.0 ± 0.8 μg L^{-1} (n = 3) in the 0.25 L reactor, indicating some initial E1 loss in the feed bottle to the 1 L reactor. The SBR operation was based on that of Dapena-Mora *et al.*[33] and López *et al.*[34] During the experiment the reactors were continuously flushed with either 95% N_2/5% CO_2 (1 L reactor) or 100% N_2 (0.25 L reactor) *via* a diffuser to maintain anaerobic conditions. A control box was used to automate the SBR sequence, which was: fill to 100% volume with synthetic influent solution over the course of 4.5 hours, react 1 hour, settle for 15 minutes, draw down to 50% volume for 10 minutes, rest for 5 minutes. The 0.25 L reactor was operated without E1 feed for approximately 60 days prior to the addition of E1 to ensure that the nitrogen removal performance was as expected and was indicative of anammox activity.

Granular activated sludge and sequencing batch reactor experiments

The granular activated sludge and standard SBR experiments were operated according to the schematic in Fig. SI-4† and the operational parameters in Table 1. The two experiments were identical except that the influent to the standard SBR experiment contained a much lower COD (200 mg L^{-1}) compared to that in the granular activated sludge experiment (1000 mg L^{-1}), resulting in sludge that failed to granulate. Operation of granular activated sludge systems differs widely;[11,12] operation of this system was focused on establishing conditions in which biomass granulated and >50% total nitrogen removal occurred. The influent, freshly prepared each day and described in the ESI† (Table SI-2), was adapted from the *Syntho* medium of Boeije *et al.*[35] and was amended with 10 μg L^{-1} E1. After amendment, E1 was measured in the influent of the granular activated sludge and SBR experiment at 12.1 ± 2.3 μg L^{-1} (n = 3) and 10.5 ± 0.6 μg L^{-1} (n = 3), respectively. Total nitrogen in the influent was approximately 86 and 57 mg L^{-1} for the granular activated

sludge and SBR experiments, respectively. As with the anammox experiment, a control box was used to automate the reactor operating sequences, which were: fill to 2 L with 1 L influent solution, react anaerobically for 2 hours, aerate for 3.5 hours, settle for 5 minutes, draw down from 2 L to 1 L. During the aeration phase, air was introduced through a disc diffuser at the bottom of the reactor column (shown in Fig. SI-4†) with an upflow velocity of about 2 cm s^{-1}. Aeration was controlled with a solenoid valve coupled to the control box. Fig. SI-5† shows a photo of the granules that formed in the granular activated sludge experiment.

Abiotic control experiments

Three negative control experiments were also performed. One experiment was operated identically to the wastewater-fed nitrification experiments, except that only tap water and E1 were fed to the reactor system; this experiment was used to determine whether E1 sorption to the membrane clarifier or other reactor materials was significant. E1 was measured in the influent at 10.5 ± 1.2 μg L^{-1} (n = 2). A second batch sorption experiment was performed to determine the extent of E1 sorption to the solids (*i.e.*, killed biomass) in the system. This experiment was performed in batch to minimize the volume of sodium azide-contaminated waste generated. In this experiment, E1 dissolved in methanol (10 μg L^{-1}) was added to triplicate 500 mL glass bottles and the methanol was allowed to fully evaporate. Mixed liquor from the Metropolitan WWTP was diluted 50% by tap water to approximate the VSS in the biologically active reactors (586 mg L^{-1}, sampled in triplicate at both time = 0 and time = 4 days), amended with sodium azide (50 mM), mixed for 24 hours, and added (250 mL per bottle) to the bottles. Samples (well-mixed) for E1 analysis were taken over a four-day period. The final abiotic control experiment was performed in a manner identical to one of the anammox experiments, with the 0.25 L reactor fed only medium and E1 and operated as an SBR. E1 was measured in the influent (10.3 ± 0.2 μg L^{-1}, n = 3) and in the reactor liquid after three consecutive react cycles (hours 6, 12, and 18).

Water quality, ammonia, and total nitrogen measurements

Volatile suspended solids (VSS), dissolved organic carbon (DOC), COD, pH, and DO were monitored as described in the ESI† (section S3). Ammonia (measured as ammonium) and total nitrogen were measured colorimetrically *via* HACH Method 10031 and HACH Method 10072, respectively. Blanks were measured during each analysis and periodic standards were measured for quality assurance. Standards averaged 103 ± 6% of expected for the total nitrogen analysis and 97 ± 2% for the ammonium analysis, agreeing well with the HACH preprogrammed calibration curve.

Nitrate (NO_3^-) and NO_2^-

NO_2^- and NO_3^- were measured in filtered (0.2 μm) samples on a 761 Compact or 930 Compact Flew Metrohm ion chromatograph outfitted with an AS-18 column and 20 μL sample loop. The eluent was 3.2 mM sodium carbonate and 1 mM sodium bicarbonate. Gravimetric standards containing sodium nitrite and sodium nitrate salts in ultrapure water were prepared to generate calibration curves with at least 6 points. Typical limits of quantification were less than 0.2 mg L^{-1} as nitrogen (mg-N L^{-1}) for both NO_3^- and NO_2^-.

E1 and 17β-estradiol (E2) analysis

Samples (10–100 mL, depending on the experiment) were collected for E1 or 17β-estradiol (E2) analysis, described in the ESI† (section S4). Solid phase extraction and clean-up procedures were adapted from Tan *et al.*[14] Additional details are provided in the ESI† (section S5). Average E1 recovery for all samples, with the exception of the influent samples to the granular activated sludge and standard SBR experiments, was 60 ± 17.5%. E1 recovery was poor, 2–17%, in the influent to the granular activated sludge and SBR experiments as a result of the high COD in the influent interfering with the SPE. One sample (one time point in one triplicate reactor) in the abiotic sorption experiment had a recovery of 1% and was therefore discarded.

An Agilent 1100 series Liquid Chromatograph (LC) with a 4000 QTRAP triple quadrupole mass spectrometer was used to measure E1 and E2. The chromatography was performed on a Synergi 4u Polar-RP 80A 150 × 2.00 mm 4 μm particle size column (Phenomenex). A binary gradient was used for compound separation. The mass spectrometer was operated in negative ion, selected reaction monitoring mode. Additional details are provided in the ESI† (section S5). Blanks of 60 : 40 methanol : water, as well as periodic method blanks were analyzed. Standard curves consisted of seven to nine external standards; an internal standard was also used (see the ESI†). Limits of quantification were 2.3 μg E1 L^{-1} solvent extract and 18.2 μg E2 L^{-1} solvent extract, which corresponds to approximately 11.5 ng E1 L^{-1} sample and 91 ng E2 L^{-1} sample. E1, E2, and the ^{13}C-labeled surrogate were corrected using the internal standard. The ^{13}C-labeled surrogate contained a significant amount of unlabeled E1, up to 6 μg E1 L^{-1} solvent extract, corresponding to 12 ng E1 L^{-1} sample. Though extremely low, given that approximately 10 μg E1 L^{-1} sample was fed to the reactors, this addition of E1 was treated similarly to a standard addition and subtracted out, based on a calibration curve developed for E1 at each surrogate recovery concentration. This curve had a limit of quantification of 0.16 to 0.3 μg E1 L^{-1} solvent extract, corresponding to a concentration of 0.3 to 0.6 ng E1 L^{-1} sample.

Data analysis

The E1 and E2 sample concentrations were calculated as follows:

$$\text{Aqueous sample concentration} = \frac{[\text{In Vial Concentration}]}{(\text{Recovery}) \times (\text{Concentration Factor})}$$

Here the concentration factor was the sample volume/solvent extract volume, and recovery was the percent surrogate recovered, expressed as a fraction.

Limits of quantification for E1, E2, ^{13}C-labeled E1, NO_2^-, NO_3^-, and DOC were produced by generating a 95% confidence interval around the calibration curve using Excel or R. The confidence interval for the lowest standard was chosen as the limit of quantification for the NO_2^-, NO_3^-, DOC and invial E1 and E2 concentrations.

Reported *p*-values were generated with R or Excel software utilizing a two-sample, two-sided, un-pooled *t* test.

NO_3^- as a percent of nitrogen removal was calculated to determine whether the nitrogen species in the effluent of the anammox reactor corresponded to the expected theoretical stoichiometry of nitrogen removal during the anammox process. Expected theoretical stoichiometry of anammox is:[36]

$$1NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0.986N_2 + 0.161NO_3^- + 0.071C_{1.74}O_{0.31}N_{0.20} + 2.002H_2O$$

Nitrogen removal occurs when N_2 is generated. Therefore, from this theoretical stoichiometry, NO_3^- as a percent of nitrogen removal is:

$$\left(\frac{0.161\ \text{mol}\ NO_3^-}{0.986\ \text{mol}\ N_2}\right)\left(\frac{1\ \text{mol}\ N_2}{2\ \text{mol}\ N_2\text{–N}}\right)\times \left(\frac{1\ \text{mol}\ NO_3^-\text{–N}}{1\ \text{mol}\ NO_3^-}\right)\left(\frac{14\ \text{g mol}\ NO_3^-\text{–N}}{14\ \text{g}\ N_2\text{–N}}\right) = 8\%$$

The corresponding value in the anammox effluent samples was calculated as follows:

$$NO_3^-\ \text{as \% of nitrogen removal} = \frac{\left[NO_3^-\text{–N}\right]_E}{\left[\text{Total N}\right]_I - \left[NH_4^+\text{–N}\right]_E - \left[NO_3^-\text{–N}\right]_E - \left[NO_2^-\text{–N}\right]_E}$$

Results and discussion

The average influent E1 concentration in these experiments was 8.4 ± 3.7 μg L^{-1} (n = 27), with the exception of the 1 L anammox experiment, in which the average influent E1 concentration was lower ($P < 0.0001$), at 4.5 ± 0.6 μg L^{-1} (n = 3), suggesting that sorption of the E1 to the feed container, or perhaps some abiotic nitration, may have occurred.[31] The overall performance of all experiments, with respect to nitrogen removal and effluent E1 concentration, is summarized in Table 2.

Abiotic E1 loss

With a log K_{OW} of 3.13,[37] E1 had the potential to sorb to the plastic feed containers, tubing, reactors, membranes, and biomass in these experiments. Abiotic E1 loss was therefore assessed. Results are shown in Fig. 1 and Table 2. Sorption to the reactor materials used in the nitrification experiments resulted in some loss of E1, on average 5 μg L^{-1}, or 46% loss of the nominally fed 10 μg L^{-1} E1. Loss appeared to stabilize rapidly in the effluent, suggesting that sorption to the tubing and membranes occurred, but reached equilibrium quickly and never decreased below about 4 μg L^{-1} E1. Very little sorption to biomass or to the anammox SBR reactor was observed (Fig. 1, Table 2).

Nitrification and MLE experiments

The conventional nitrification and MLE experiments performed as expected with respect to nitrogen removal (Table 2, Fig. SI-6†). In the nitrification experiments, complete NH_4^+ removal was achieved by day 17, at which point approximately 62% of the influent NH_4^+ was converted to NO_3^-. After day 24, the MLE reactor stabilized at approximately 68% total nitrogen removal with a NO_3^--rich effluent (Table 2, Fig. SI-6†). Based on an internal recycle rate of 2Q, 67% total nitrogen removal was expected.[38]

As expected based on the literature,[14,15,21–23] E1 removal was also excellent in the nitrification and MLE experiments (Table 2, Fig. SI-7†). In the nitrification experiment, E1 was removed to <0.44 μg L^{-1} E1 throughout the experiment (Fig. SI-7†), which was significantly different than the effluent E1 concentration in the abiotic experiments ($P < 0.0001$). Similarly, in the MLE experiment E1 was present in the effluent at a concentration <0.42 μg L^{-1} throughout the experiment (Fig. SI-7†); again, significantly different from the effluent E1 concentration in the abiotic experiments ($P < 0.0001$). Assuming a nominal influent E1 concentration of 10 μg L^{-1}, both the conventional nitrification and MLE systems were capable of approximately 98% E1 removal.

These conventional nitrification and nitrification/denitrification technologies were expected to degrade E1 effectively as a result of the reactor conditions: low, consistent concentrations of dissolved organic carbon, constant aeration during nitrification, and a long solids residence time.[14,23,26] The removal performance observed in these experiments was also consistent with that observed in the literature.[15,39] Suarez *et al.*[39] utilized side-by-side nitrifying and denitrifying lab scale treatment systems to assess estrogen and personal care product removal. Excellent (99%) removal of E1 + E2 was observed in the aerobic nitrifying treatment system and good (72%) removal of the same was observed in the denitrifying treatment system. Analysis of a German full-scale plant also noted 98% removal of E1 + E2 after treatment with conventional nitrification and denitrification combined with phosphate removal.[15]

Anammox experiment

Strong evidence for anaerobic ammonia oxidation, in addition to excellent E1 removal, was observed during the

Table 2 Performance after days 3, 17, 13, 25, and 20 in the abiotic controls, nitrification, MLE, granular activated sludge, and sequencing batch reactor experiments, respectively. Anammox data is for the stable period between days 23 and 40

	Effluent DOC (mg L^{-1})	Reactor liquor VSS (mg L^{-1})	Total nitrogen removal (%)	Effluent [E1] (μg L^{-1})
Abiotic control clean water	NA[a]	NA	NA	4.9 ± 1.2 (n = 4)
Abiotic control Azide-killed	NA	586 ± 73 (n = 6)	NA	9.4 ± 1.9 (n = 11)
Abiotic SBR control	NA	NA	NA	10.6 ± 0.4 (n = 3)
Nitrification	10.5 ± 1.2 (n = 8)	710 ± 150 (n = 8)	38 ± 7 (n = 3)	0.26 ± 0.11 (n = 14)
MLE	11.9 ± 1.3 (n = 6)	300 ± 129 (n = 6)	68 ± 7 (n = 6)	0.23 ± 0.13 (n = 7)
Anammox (1 L)	14.5 ± 3.0 (n = 6)	200 ± 190 (n = 6)	77 ± 7 (n = 6)	0.01 ± 0.01 (n = 5)
Anammox (0.25 L)	n.d.[b]	1793 ± 140 (n = 3)	69 ± 16 (n = 11)	1.40 ± 0.36 (n = 3)
Granular activated sludge	31.3 ± 8.8 (n = 15)	n.d.[b]	73 ± 5 (n = 15)	10.4 ± 3.9 (n = 8)
Sequencing batch reactor	3.2 ± 0.8 (n = 12)	n.d.	27 ± 7 (n = 12)	0.44 ± 0.30 (n = 6)

[a] NA indicates that this parameter is not applicable. [b] n.d. indicates that this parameter was not determined.

laboratory-scale anammox experiment (Fig. SI-8, 2,† and Table 2). Based on stoichiometry, if a pure culture of anaerobic ammonia oxidizing organisms are present, the proportion of produced NO_3^-–N to the total nitrogen removed should be equal to 8% (see Data analysis).[36] The NO_3^- concentration as a percent of total nitrogen removed was near this value (12.4 ± 5% after day 2 in the first 1 L anammox experiment and 7.7 ± 3% after day 19 for the second 0.25 L experiment) (Fig. SI-8†). Between days 23 and 40 in the first experiment and days 19 and 67 in the second experiment total nitrogen removal ranged from 45–89% of the total influent nitrogen (Fig. 2), which agreed well with a similar laboratory-scale SBR study in which the average nitrogen removal was 78%.[33] A reactor upset, evidenced by floating biomass, NO_2^-–N accumulation to between 25 and 50 mg L^{-1}, and decreased total nitrogen removal efficiency, occurred between days 13 and 20, and on the final day of the first experiment (day 42) (Fig. 2A). Throughout the two anammox experiments, the effluent E1 concentration was 0.5 ± 0.7 μg L^{-1} (Fig. 2, Table 2), with 0.01 ± 0.01 μg L^{-1} effluent E1 in the 1 L reactor and 1.40 ± 0.36 μg L^{-1} effluent E1 in the 0.25 L reactor. These values were significantly different than the effluent E1 concentration in the abiotic experiments ($P < 0.0001$). Assuming a nominal influent E1 concentration of 10 μg L^{-1}, this corresponded to an average removal of 89–99.7% E1. Because the E1 concentrations in the influent were stable at 4.5 ± 0.6 μg L^{-1} (n = 3) and 13.0 ± 0.8 μg L^{-1} (n = 3) for the 1 L and 0.25 L experiments, respectively, abiotic E1 nitration was not responsible for the low effluent E1 concentrations.[31] In the second anammox experiment, both E1 and E2 were monitored in the effluent and E1 transformation to E2 was not observed, with influent E2 concentrations (likely as a result of impurities in the E1 feed) of 0.10 ± 0.01 (n = 3) and effluent E2 concentrations of 0.24 ± 0.14 (n = 3). These values were not significantly different from one another (P = 0.22).

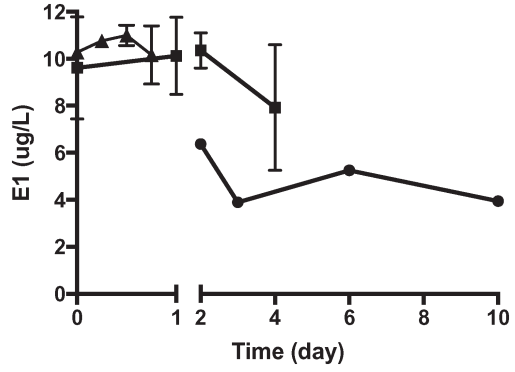

Fig. 1 E1 concentrations in the control experiments. Filled circles (●) show E1 effluent concentrations in the clean water flow-through control experiment. Closed squares (■) show E1 concentrations in the azide-killed biomass batch experiments. Closed triangles (▲) show E1 concentrations in the clean water SBR control experiment.

The excellent E1 removal observed during the anammox experiments was somewhat unexpected. E1 degradation is typically associated with aerobic systems with low organic carbon concentrations and a long SRT.[24,26,39] E1 degradation was observed in a nitritation/anammox process[29] and in a recent study assessing the degradation of E2 and E1 during the anammox treatment of synthetic urine.[30] Nevertheless, experiments assessing the potential of E1 to degrade under anaerobic conditions with anaerobic digester sludge, activated sludge, and upflow anaerobic digester sludge showed little potential for E1 to degrade.[40] It was therefore assumed that E1 degradation might not occur, or would be slow, as was recently observed in an anammox experiment.[30] Excellent and rapid E1 degradation did occur without concomitant E2 formation, however. In this experiment, the long SRT may have allowed anaerobic E1 degraders, such as E1-utilizing denitrifiers,[27,28] to grow in the initial sludge sample and begin degrading E1 soon after experiment initiation. Alternatively, the anammox microorganisms themselves may have played a part in E1 degradation.

Granular activated sludge and standard SBR experiments

In the laboratory-scale granular activated sludge experiment, stable granules formed (Fig. SI-5†), 61–87% of the influent nitrogen was removed, and nitrogen removal was stable (Table 2). The performance of this system agreed well with the total nitrogen removal reported in a continuously aerated granular activated sludge experiment (65–82%)[41] and in a full-scale granular activated sludge plant (60%).[42] Effluent DOC averaged 31.3 ± 8.8 mg L^{-1} (Table 2), showing that

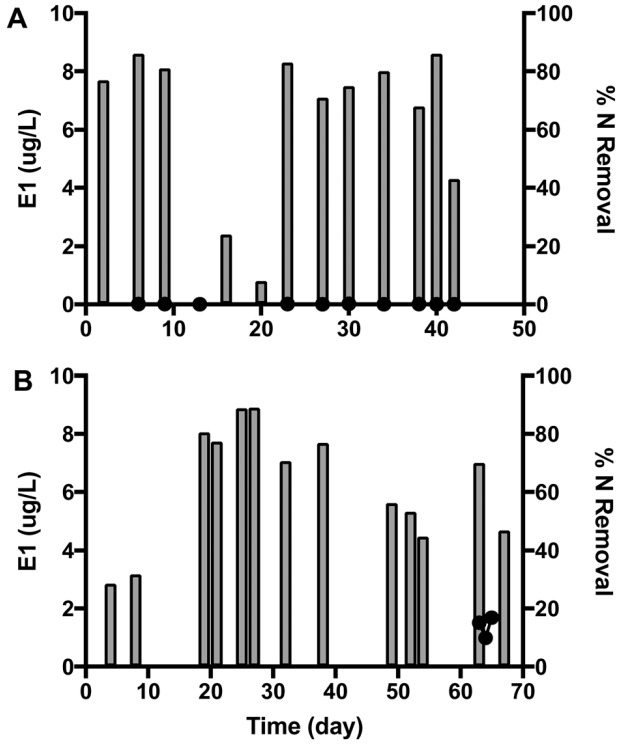

Fig. 2 E1 and total nitrogen removal in the anammox experiments, with (A) showing results from the 1 L reactor and (B) showing results from the 0.25 L reactor. Here, closed circles (●) show effluent E1 concentrations and grey bars show percent total nitrogen (%N).

stable overall carbon removal >90% occurred throughout the experiment as well. Unlike the other reactor systems investigated, the granular activated sludge system provided only very limited E1 removal, with an average effluent E1 concentration of 10.4 ± 3.9 μg L^{-1} after day 25 (Table 2, Fig. 3). This was not significantly different than the effluent concentration measured in the abiotic experiments (P = 0.26). It is possible that high influent COD concentrations select for fast-growing bacteria at and near the surface of the granules, where aerobic conditions existed. This would select against slower-growing E1 degrading microorganisms, as has been observed by others.[14,24] Alternatively, abundant electron donor could have altered the expression of metabolic pathways, switching off "scavenging" functions and as a result, hindering E1 removal.[24] A third, albeit unlikely possibility is that the reactor configuration had a negative impact on E1 removal.

To clarify the importance of high COD concentrations *versus* reactor configuration, an additional SBR experiment was performed, identical to the granular activated sludge experiment except that the influent COD was lowered from 1 g L^{-1} to 200 mg L^{-1} COD. No granule formation was observed in this experiment, and while organic carbon was degraded effectively, nitrogen removal was negatively affected (Table 2, Fig. 3), likely because of the short settling time. Nevertheless, with the lower influent COD concentration and all other operating parameters identical, E1 removal was excellent, unlike the removal observed in the granular activated sludge experiment. Indeed, the effluent E1 concentrations in this SBR experiment were ≤0.82 μg L^{-1} E1 after day 20, significantly differing from the effluent E1 concentrations in the abiotic experiments ($P < 0.0001$) (Fig. 3). If a nominal influent E1 concentration of 10 μg L^{-1} is assumed, this corresponds to an average E1 removal of 96%. This level of E1 removal was similar to that observed during the nitrification, MLE, and anammox experiments. It is therefore likely that the lower COD concentrations allowed E1 degraders to compete and grow despite the loss of nitrogen removal capacity. As stated above, granulation did not occur in this reactor without high influent COD concentration and loading. Others, however, have observed granulation at lower influent COD concentrations and loadings;[12] therefore, it is possible that under those conditions E1 degradation would occur in a granulated system. The excellent E1 removal performance observed in the standard SBR experiment agreed with results from the literature in which excellent (60–90%) E1 removal was observed in similarly operated NO_2^--accumulating SBRs.[43]

Environmental implications and extrapolation of results

The degradation of E1 is only beneficial if its degradation products no longer contribute to the estrogenicity of the

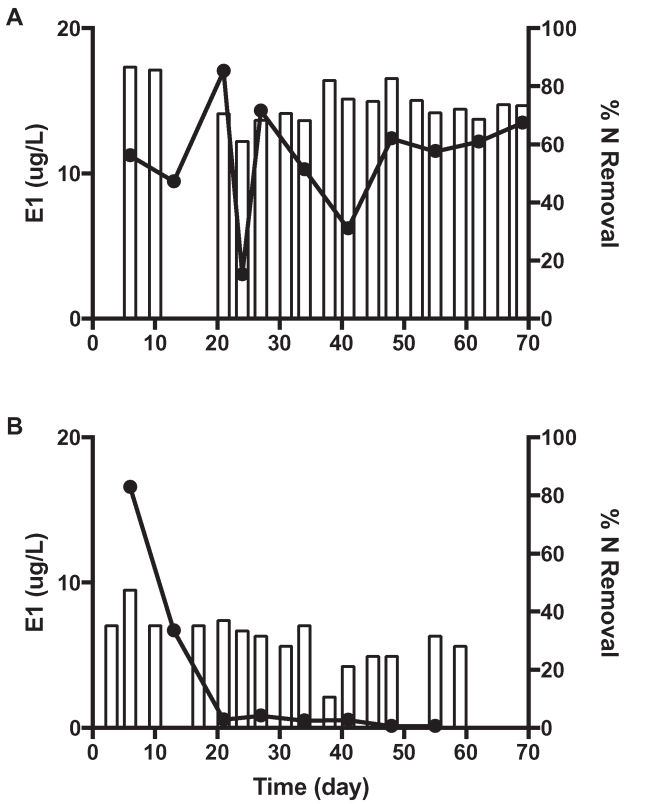

Fig. 3 Effluent E1 concentrations and total nitrogen removal in the granular activated sludge (panel A) and sequencing batch reactor (panel B) experiments. Here, solid lines with closed circles (●) show effluent E1 concentration and bars show percent nitrogen (%N) removed.

effluent. In conventional aerobic systems this is a reasonable assumption based on the current literature, where estrogen degradation in aerated activated sludge has been associated with decreased estrogenicity. In one study,[44] 95–100% removal of total estrogens in a conventional aerated full-scale WWTP operated with nutrient removal corresponded to 87–99% removal of estrogenicity. In another study,[45] the aerated stage of treatment in a survey of five WWTPs was associated with the greatest decrease in estrogenicity. These results indicate that the removal of E1 in conventional nitrification and nitrification–denitrification processes is likely to be associated with a corresponding removal of estrogenicity. This coupled removal of E1 and estrogenicity cannot be assumed, however, in the absence of oxygen, such as in an anammox process. Indeed, the products of E1 transformation, although shown not to be E2, were unidentified in our experiments. E1 has also been shown to slowly transform to unknown products during anammox treatment of synthetic urine.[30] Further study is needed to determine the products associated with E1 removal in this system and to determine whether the removal of estrogenicity occurs as well.

In this study, the nitrogen removal technologies that were capable of E1 removal shared one trait: the presence of low organic carbon concentrations. Effluent DOC was about 10 to 15 mg L^{-1} in the nitrification, MLE, and anammox reactor systems, indicating that organic carbon was present in each system, but at relatively constant, low concentrations. The presence of low concentrations of organic carbon combined with a long SRT should stimulate the growth of multiple substrate degrading heterotrophs, the microorganisms implicated in E1 degradation in aerated systems.[14] The influent to the aerobic granular sludge reactor operated in this research contained a high COD, consisting of entirely soluble synthetic wastewater constituents (Table SI-2†), which likely fostered the rapid growth of aerobic heterotrophs. Although the effluent COD was relatively low (approximately 30 mg L^{-1}), the feast-famine conditions in this reactor either selected against slower growing E1-degrading microorganisms[14,24] or altered the expression of the "scavenging" functions of E1-degrading multiple substrate utilizers.[24] It is possible that granular activated sludge systems operated differently[12] could degrade E1, or under certain conditions denitrifiers capable of growth on E1 could be active.[27,28] Nevertheless, nitrogen removal technologies that result in the presence of low and relatively constant concentrations of organic carbon are more likely to remove E1 than those technologies in which high, or highly fluctuating, concentrations of organic carbon are present.

If there is a need for total nitrogen removal and concerns about effluent estrogen, such as at plants that discharge to effluent-dominated receiving bodies, low energy treatment options do exist. Indeed, E1 removal was comparable during MLE and anammox treatment. Anammox has an advantage over conventional nitrogen removal in that the partial nitritation phase consumes much less oxygen than conventional nitrification, and therefore, much less energy.[9] This offers a distinct advantage if the anammox process can be reliably mainstreamed. This work demonstrated that anammox technology can remove an important estrogenic contaminant, E1, while also effectively removing total nitrogen. As long as the degradation products of E1 produced during anammox are not harmful and estrogenic, anammox treatment has the potential to be a practical and effective treatment method for wastewaters that are both nitrogen-rich and estrogenic. Finally, given that implementation of anammox requires aeration to accomplish partial nitritation, E1 removal would likely be further enhanced in such a process.

Acknowledgements

The work was supported by the Environment and Natural Resources Trust Fund as recommended by the Legislative Citizen Commission on Minnesota Resources. D. T. and J. C. B.-C. were funded by MnDRIVE postdoctoral fellowships. The authors would like to thank Sarah Barnett for assistance in the laboratory.

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