Supporting Information

Estrone Biodegradation in Laboratory-Scale Systems Designed for Total Nitrogen Removal from Wastewater

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Section S1. Reactor Seed

To prepare the concentrated activated sludge aliquots for use in the experiments, six 500 mL portions of activated sludge supernatant was centrifuged for 15 minutes at 5000 rpm and the resulting supernatant was decanted and replaced with phosphate buffer. The centrifugation followed by phosphate buffer addition process was repeated two more times, with the resulting concentrate combined with enough phosphate buffer to resuspend the solids. The resulting suspension was preserved in a 15% glycerol solution and divided into 10 mL aliquots prior to freezing at -20 °C until use.

Section S2. Experimental Set-up

Reactor schematics are shown in Figures SI-1, SI-2, SI-3, and SI-4 for the nitrification, MLE, anammox, and granular activated sludge experiments, respectively.

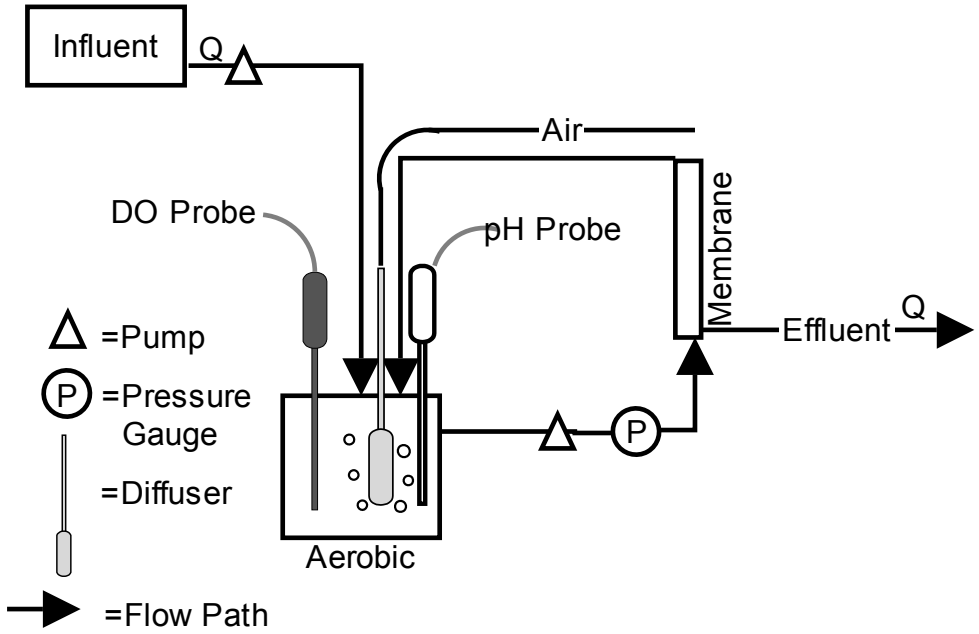

Figure SI-1. Schematic of the nitrification reactor set-up.

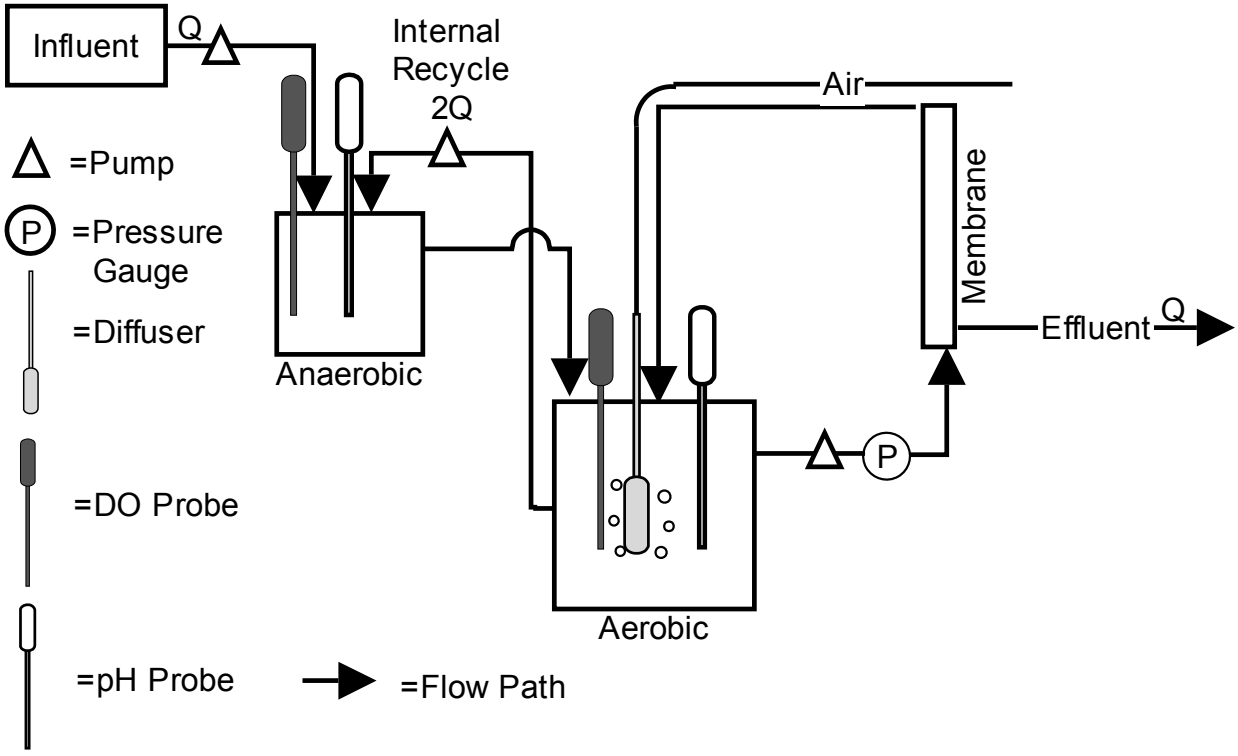

Figure SI-2. Schematic of the MLE reactor set-up.

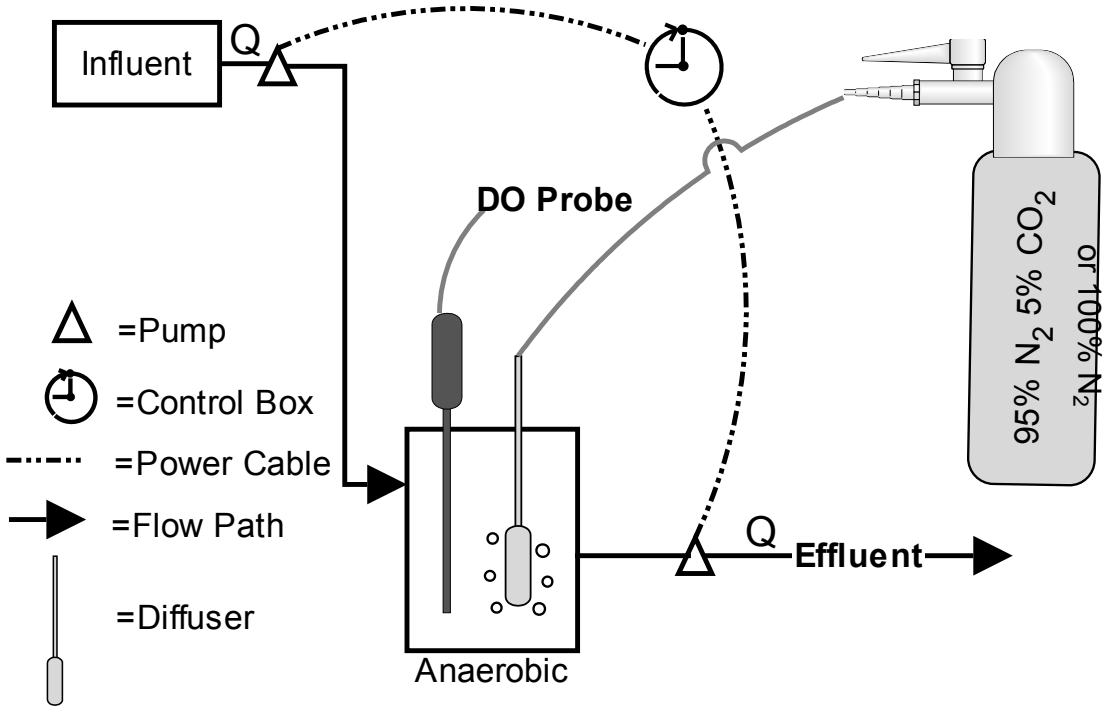

Figure SI-3. Schematic of the anammox reactor set-up.

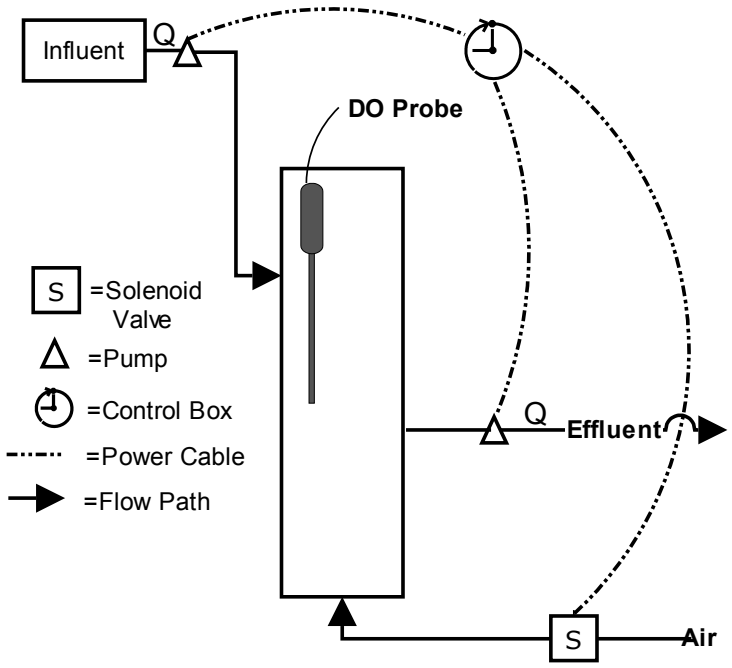

Figure SI-4. Schematic of the granular activated sludge reactor set-up.

The anammox feed, adapted from Van de Graaf et al.,[1] was replaced every 48 hours and contained the following per 1 L of deionized water: 1 mL trace solution 1, 1 mL trace solution 2, 1 mL Mg solution, 1 mL Ca solution, 27.2 mg KH_2PO_4 and 500 mg $KHCO_3$. The composition of the trace solutions, Mg Solution and Ca solution are given in Table SI-1. The total N added to the reactor was approximately 130 mg/L N between days 0-20 (330 mg $(NH_4)_2SO_4$ and 345 mg $NaNO_2$). Following an upset on Day 20 of the first (1-L) anammox experiment, an additional 100 mL of the DEMON sludge was added to the reactor and the influent total N was decreased to approximately 100 mg/L (254 mg $(NH_4)_2SO_4$ and 265 mg $NaNO_2$).

Table SI-1. Composition of the solutions used to prepare the anammox synthetic influent.

Solution	Chemical	Mass (g) Added Per 1 L of Deionized Water
Trace Solution 1	EDTA	5
	$FeSO_4 \cdot 7H_2O$	9.1
Trace Solution 2	EDTA	7.5
	$ZnSO_4 \cdot 7H_2O$	0.215
	$CoCl_2 \cdot 6H_2O$	0.12
	$MnCl_2 \cdot 4H_2O$	0.495
	$CuSO_4 \cdot 5H_2O$	0.125
	$NaMoO_4 \cdot 2H_2O$	0.11
	Na_2SeO_4	0.054
	$NiCl_2 \cdot 6H_2O$	0.095
	H_3BO_3	0.007
Mg Solution	$MgSO_4 \cdot 6H_2O$	30.6
Ca Solution	$CaCl_2$	13.6

The influent for the granular activated sludge experiment and sequencing batch reactor experiment was adapted from the *Syntho* medium of Boeije et al.[2] The composition of the influent is given in Table SI-2.

Table SI-2. Influent composition used in the granular activated sludge and sequencing batch reactor experiments; added per 1 liter deionized water.

	Granular Activated Sludge Influent (1000 mg/L COD)	Sequencing Batch Reactor Influent (200 mg/L COD)
Chemical	Mass (mg)	Mass (mg)
Urea	75.0	75.0
NH_4Cl	11.0	11.0
Sodium Uric Acid	12.0	12.0
$MgHPO_4 \bullet 3H_2O$	25.0	25.0
K_3HPO_4	20.0	20.0
Bacteriological Peptone	64.0	12.8
Sodium Acetate Trihydrate	849	170
Dry Meat Extract	64.0	12.8
Potato Starch	320	42.7
Skim Milk Powder	254	50.8
Glycerol	171	34

Figure SI-5 shows granules that formed in the granular activated sludge reactor.

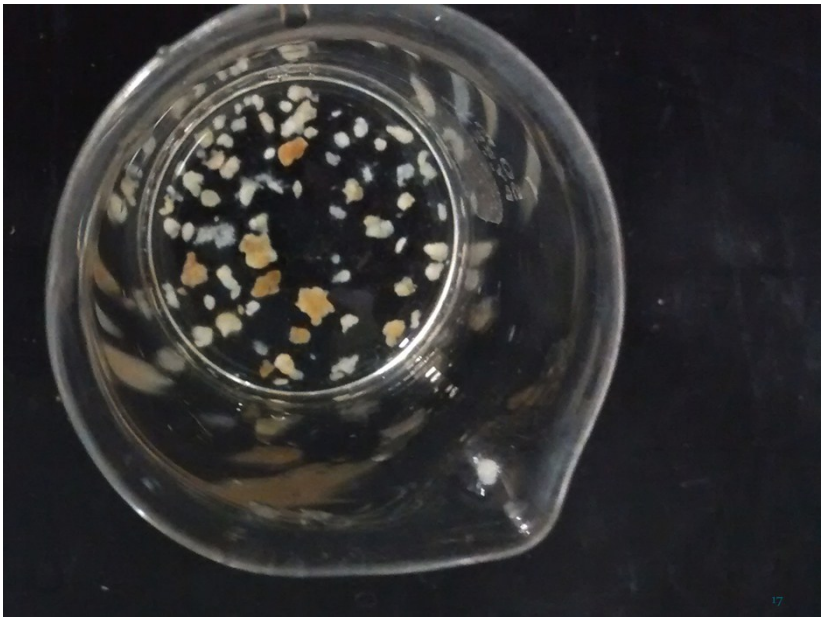

Figure SI-5. Photograph of the granules (approximately 2-4 mm in diameter) from the granular activated sludge experiment, as viewed from above in a beaker.

Section S3. Water Chemistry and Reactor Effluent Measurements

To measure VSS, a volume of mixed liquor was filtered through an ashed Whatman® Glass microfiber filter Grade GF/A. The filter was dried at 105 °C overnight, weighed, ignited at 550 °C overnight, and weighed a second time. VSS was calculated from the absolute difference between the dry and ignited weights divided by the volume of the filtered sample. DOC was measured in filtered (0.2 μm) effluent samples using a Shimadzu TOC-L total organic carbon analyzer on the non-purgeable organic carbon setting. A stock solution of hydrogen phthalate was diluted by the instrument to generate seven-point calibration curves from 2.5 to 25 mg/L C or 10 to 50 mg/L C, as appropriate. Typical limits of quantification were less than 2 mg/L C. COD was measured via the colorimetric HACH Method 8000. One 200 mg/L (as C) hydrogen phthalate

check standard was measured for quality assurance; it was measured at 99% of nominal, agreeing well with the HACH preprogrammed calibration curve. pH and DO were monitored continuously every few minutes via a data logger coupled to a Vernier pH sensor and a Vernier optical DO probe in both the nitrification and MLE experiments. DO was monitored via data logger and a Vernier optical DO probe seven times per hour in the granular activated sludge and sequencing batch reactor experiments. DO was measured once per hour with a WTW FDO® 925-3 DO probe in the anammox experiment. pH was monitored manually at periodic intervals during the granular activated sludge, sequencing batch reactor, and anammox experiments.

Section S4. E1 and E2 Measurement: Sample Collection, Extraction, and Clean-up Procedures

All samples, excluding the effluent samples from the granular activated sludge, sequencing batch, and anammox reactors, were taken and extracted with no sample manipulation. Given the nature of the experimental set-up, the effluent samples from the nitrification and MLE reactors had passed through the membrane prior to collection; therefore, they were essentially filtered (0.2 μm) prior to extraction. Effluent samples from the granular activated sludge and sequencing batch reactor experiments were acidified to a pH <3 with concentrated sulfuric acid prior to sample extraction. Effluent samples from the anammox experiment were pH-adjusted in a manner identical to that used for the granular activated sludge and sequencing batch reactor samples, but before sample extraction they were also frozen at -18°C, then allowed to return to room temperature and pH-adjusted to near 6. Acidification and cryopreservation has been shown to be an effective preservation and storage method for estrogen samples if samples are not immediately extracted.[3] The additional pH adjustment to near neutral in the anammox effluent samples prior to extraction was necessary for good E1 recovery.

Solid phase extraction and clean-up procedures were adapted from Tan et al.[4] Known sample volumes ranging from 10 mL to 100 mL were amended with 5 μL of a labeled surrogate

in methanol (13,14,15,16,17,18-^{13}C6-estrone). Two column volumes of acetone followed by two column volumes of water were used to condition a Resprep Bonded Reversed Phase SPE cartridge (Restek). Samples were added to the cartridges at a flow rate of approximately 3 mL/min and were eluted with two column volumes of acetone. Following elution, samples were dried under a steady stream of N_2 and suspended in 3 mL of hexane before loading on silica gel columns. Silica gel columns were prepared in Pasteur pipets stopped with glass wool, packed with 2.5 cm of silica gel and washed with 6 mL hexane. The columns were eluted with 5 mL of a 65% acetone/35% hexane solvent mixture (v/v) and dried under a steady stream of nitrogen. Samples were resuspended in a 60:40 mixture of methanol and water (v/v) with 5 μL of an internal standard in methanol (2,4,16,16-D_4-estrone) and stored at 4°C until analysis. Average recovery for all samples, with the exception of the influent samples to the granular activated sludge and sequencing batch reactor experiments, was 60±17.5%. The recovery in the granular activated sludge and sequencing batch reactor experiment influent samples was low as a result of the high soluble COD in these samples, at 2-17% and should therefore be viewed with caution.

Section S5. E1 and E2 Measurement: Separation and Quantification

A binary gradient of solutions A and B with a flow rate of 0.2 mL/min was used for compound separation. Solution A consisted of a 90% water 10% acetonitrile solution with 2 mM ammonium acetate; solution B consisted of 100% acetonitrile. From 0-2 minutes 30% solution B was used; from 2-10 minutes there was a linear increase in solution B to a final percentage of 95%. Solution B remained at 95% from minutes 10-11, at which point it dropped to 30% from minute 11 to minute 17.

The mass spectrometer was operated in negative ion, selected reaction monitoring mode. Table SI-3 shows the pairs of *m*/*z* ratios chosen for quantification and confirmation. Q indicates the pair used for quantification, C indicates the pair used for confirmation.

Table SI-3. *m/z* ratios used for E1 and E2 quantification and confirmation.

Analyte	E1	E2	Internal Standard	Surrogate
***m*/*z* pairs**	Q[a]: 269-145 C[b]: 269-143	Q: 271-145 C: 271-183	Q: 273-147 C: 273-187	Q: 275-145 C: 275-186

[a]*m/z* ratio used for quantification.

[b]*m/z* ratio used for confirmation.

Section S6. Additional Results

NH_4^+, NO_2^- and NO_3^- concentrations as a function of time in the nitrification and MLE experiments are shown below in Figure SI-6.

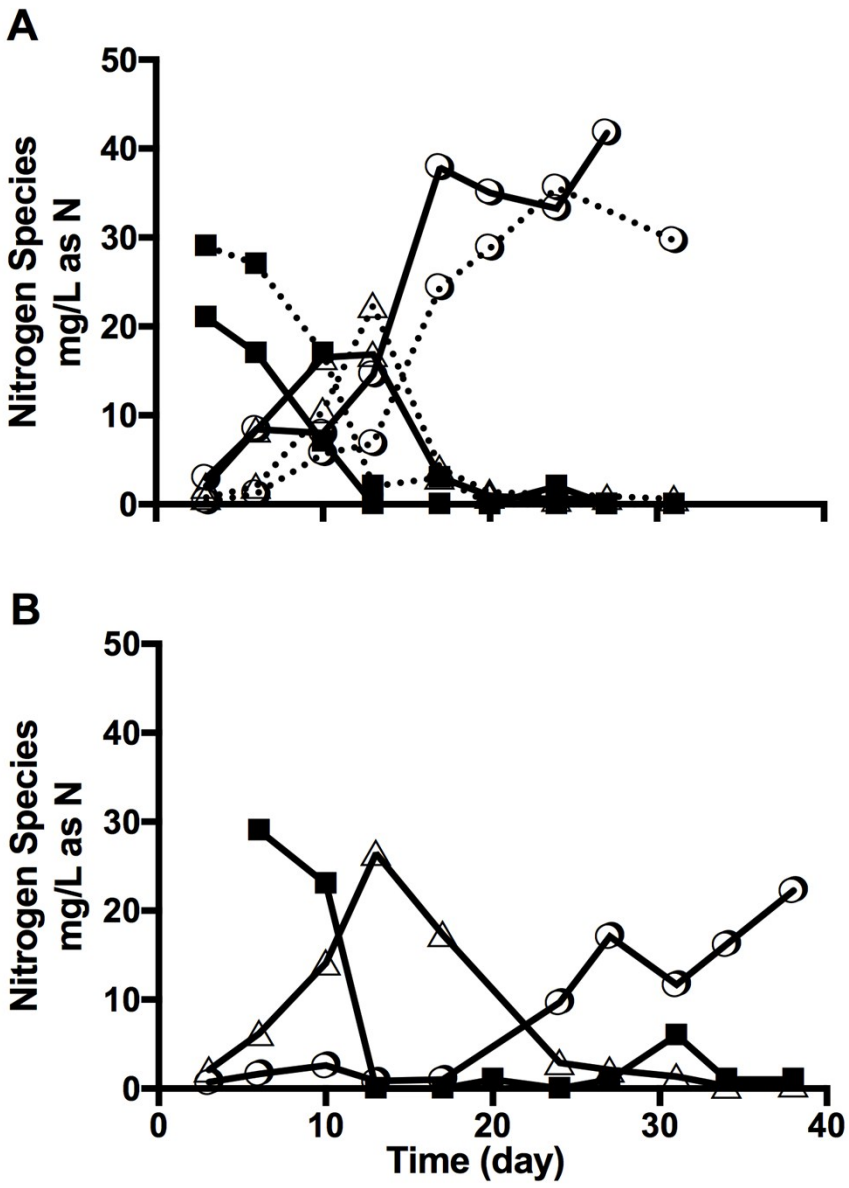

Figure SI-6. NH_4^+, NO_2^- and NO_3^- concentrations as a function of time in the nitrification (Panel A) and MLE (Panel B) experiments. Here dotted lines and solid lines show results from replicate experiments. Closed squares (■) are NH_4^+, open circles (○) are NO_3^-, and open triangles (△) are NO_2^- concentrations.

E1 removal in the nitrification and MLE experiments as a function of time is shown below in Figure SI-7.

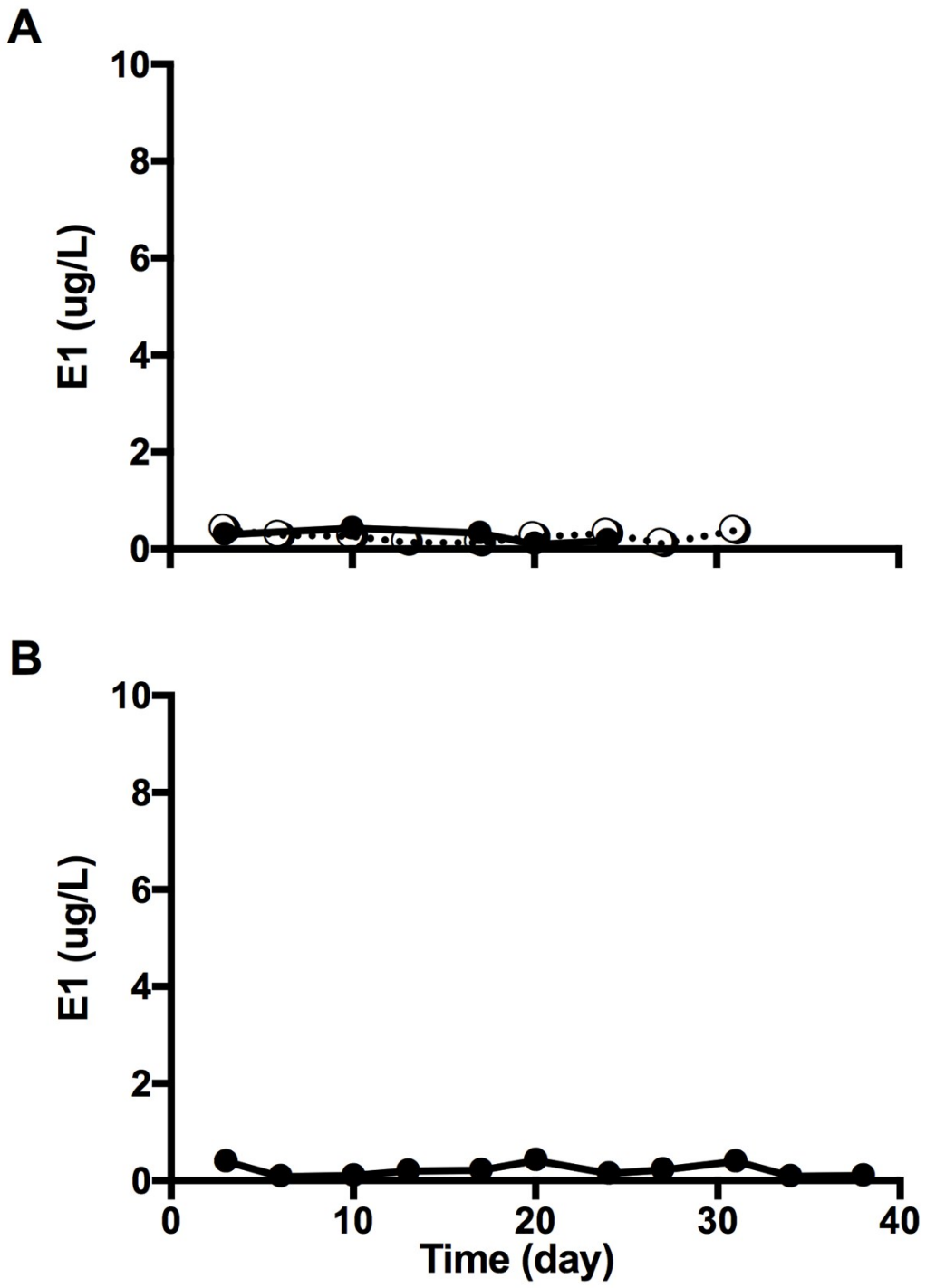

Figure SI-7. Effluent E1 in the nitrification (Panel A) and MLE (Panel B) experiments. Here, solid lines with closed circles (ꕥ) and dotted lines with open circles (ꕥ) show results from replicate experiments.

The removal and production of N-species in the anammox reactor indicated that anaerobic ammonia oxidation was active (Figure SI-8), with an average NO_3^- concentration as a percent of total nitrogen removed of 12.4±5% after Day 2 of the first (1-L) experiment and 7.7±3% after Day 19 for the second 0.25-L experiment.

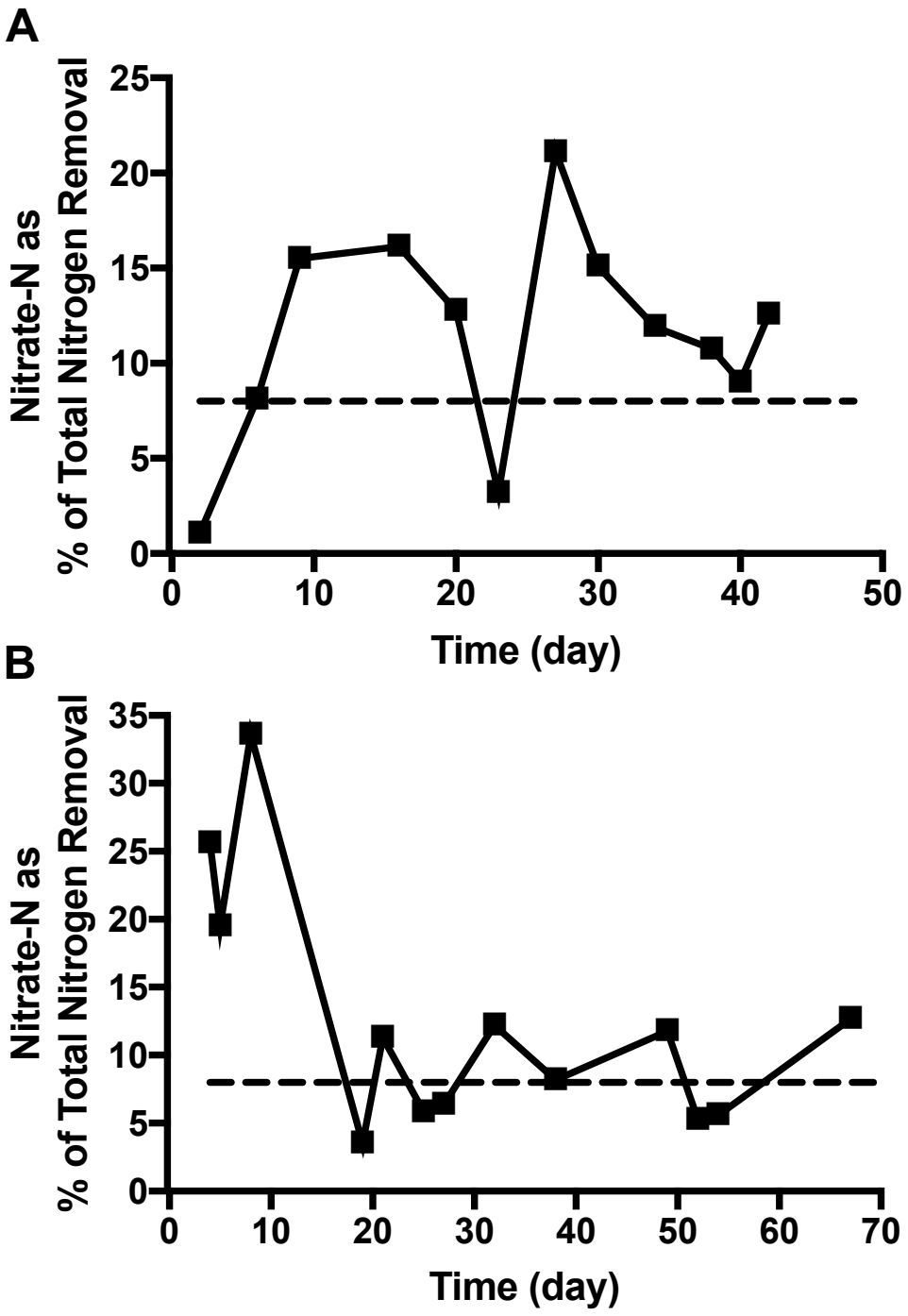

Figure SI-8. NO_3^--N as a percentage of the total N removed during the anammox experiment in the1-L SBR (Panel A) and 0.25-L SBR (Panel B). The dotted line represents the theoretical stoichiometric quantity of NO_3^--N (8%) that should be present as a percentage of the total nitrogen removal.[5] Note, E1 was added over Days 6-40 in the 1-L anammox experiment and 63-65 in the 0.25-L anammox experiment.

References

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(3) Raman, D.R.; Layton, A.C.; Moody, L.B.; Easter, J.P.; Sayler, G.S.; Burns, R.T.; Mullen, M.D. Degradation of estrogens in dairy waste solids: effects of acidification and temperature. *Trans. ASAE* **2001,** *44*, 1881-1888.

(4) Tan, D.T.; Arnold, W.A.; Novak, P.J. Impact of organic carbon on the biodegradation of estrone in mixed culture systems. *Environ. Sci. Technol.* **2013,** *47*, 12359–12365.

(5) Lotti, T.; Kleerebezem, R.; Lubello, C.; van Loosdrecht, M.C.M. Physiological and kinetic characterization of a suspended cell anammox culture. *Water Res*. **2014,** *60*, 1-14.