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Supporting information for:

**Integrated Low-Energy and Low Carbon Shortcut Nitrogen removal with Biological  
Phosphorus Removal for Sustainable Mainstream Wastewater Treatment**

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Yang, Joseph A. Kozak, Heng Zhang, George F. Wells

8 **S1. Methods**

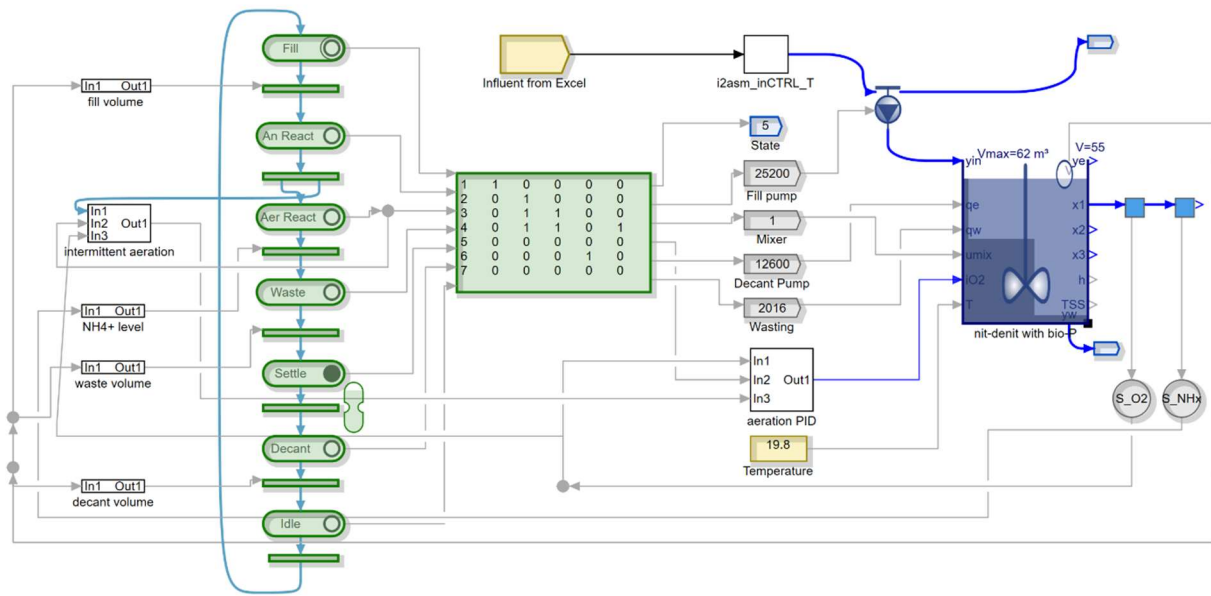
9 **S1.1. Aeration control**

10 The variable-length aerated react period was terminated if either a maximum allowable react  
11 time was reached (usually set between 300 – 480 minutes) or if the target  $\text{NH}_4^+$  concentration (3 –  
12 5  $\text{mgNH}_4\text{-N/L}$  in Phase 1, 2  $\text{mgNH}_4\text{-N/L}$  from days 247 - 430 and 1.5  $\text{mg NH}_4\text{-N/L}$  from days 431  
13 - 531 in Phase 2) was reached according to the online ammo::lyser™ ion-selective electrode  
14 (s::can, Vienna, Austria). Intermittent aeration was used during the aerated react period with the  
15 following loop:

- 16 1. 4 or 5 minutes of aeration with proportional-integral (PI) control to target 1  $\text{mgO}_2\text{/L}$   
17 via the online dissolved oxygen (DO) oxi::lyser™ optical probe (s::can, Vienna,  
18 Austria). PI control managed the percent-open time of an air solenoid valve, which,  
19 when open, provided compressed air at 7 – 15 liters per minute through a 5-inch  
20 diameter aquarium stone disk diffuser at the bottom of the reactor.
- 21 2. After aeration, shut air solenoid valve and wait until DO drops to  $< 0.05 \text{ mgO}_2\text{/L}$ .
- 22 3. Run “anoxic” timer for 0 – 3 minutes. At end of timer, return to Step 1.

23 Due to variable oxygen uptake rates (OUR) and changes to the anoxic timer, the overall  
24 aerobic/anoxic interval lengths typically varied between 10 – 20 minutes.

25 **S1.2 Process Modeling**



26

27 **Figure S1.** Process representation in the Simba# 3.0 software.

28

29 Modeled specific growth rates for AOO, NOO, and PAOs were quantified throughout the  
 30 SBR cycles with rate equations and parameter values from the Simba# inCTRL ASM matrix. Rate  
 31 equations and parameters values (at 20°C) discussed in the text are as follows:

32 ***net specific growth rate of AOO ( $d^{-1}$ ) =  $\mu_{AOO}$***

33 
$$= \hat{\mu}_{AOO} \frac{S_{NHx}}{S_{NHx} + K_{NHx,AOO}} \frac{S_{O2}}{S_{O2} + K_{O2,AOO}} \frac{S_{PO4}}{S_{PO4} + K_{PO4,ANO}} \frac{S_{ALK}}{S_{ALK} + K_{ALK,AOO}}$$

34 
$$- \hat{b}_{AOO,O2} \frac{S_{O2}}{S_{O2} + K_{O2,AOO}} - \hat{b}_{AOO,NOx} \frac{S_{NO3} + S_{NO}}{S_{NO3} + S_{NO2} + K_{NOx,ANO}} \frac{K_{O2,AOO}}{S_{O2} + K_{O2,AOO}}$$

35 
$$- \hat{b}_{AOO,ANA} \frac{K_{NOx,ANO}}{S_{NO3} + S_{NO2} + K_{NOx,ANO}} \frac{K_{O2,AOO}}{S_{O2} + K_{O2,AOO}}$$

36 Where:

37  $\hat{\mu}_{AOO}$  = maximum specific growth rate of AOO ( $d^{-1}$ ) = 0.9

38  $S_{NHx}$  = concentration of  $NH_4^+ + NH_3$  ( $\frac{mgN}{L}$ )

39  $K_{NHx,AOO}$  = AOO half saturation coefficient for  $(NH_4^+ + NH_3)$  ( $\frac{mgN}{L}$ ) = 0.7

40  $S_{O_2} = \text{concentration of dissolved } O_2 \left( \frac{mgO_2}{L} \right)$

41  $K_{O_2,AOO} = AOO \text{ half saturation coefficient for dissolved } O_2 \left( \frac{mgO_2}{L} \right) = 0.25$

42  $S_{PO_4} = \text{concentration of } PO_4^{3-} \left( \frac{mgP}{L} \right)$

43  $K_{PO_4,ANO} = \text{nitrifier nutrient half saturation coefficient for } PO_4^{3-} \left( \frac{mgP}{L} \right)$

44  $= 0.001$

45  $S_{ALK} = \text{concentration of alkalinity} \left( \frac{meq}{L} \right)$

46  $K_{ALK,AOO} = AOO \text{ half saturation coefficient for alkalinity} \left( \frac{meq}{L} \right) = 0.5$

47  $\hat{b}_{AOO,O_2} = \text{maximum specific aerobic decay rate of AOO} (d^{-1}) = 0.17$

48  $\hat{b}_{AOO,NOx} = \text{maximum specific anoxic decay rate of AOO} (d^{-1}) = 0.1$

49  $S_{NO_3} = \text{concentration of } NO_3^- \left( \frac{mgN}{L} \right)$

50  $S_{NO_2} = \text{concentration of } NO_2^- \left( \frac{mgN}{L} \right)$

51  $K_{NOx,ANO} = \text{nitrifier half saturation for anoxic conditions} \left( \frac{mgN}{L} \right) = 0.03$

52  $\hat{b}_{AOO,ANA} = \text{maximum specific anaerobic decay rate of AOO} (d^{-1}) = 0.05$

53

54 **net specific growth rate of NOO ( $d^{-1}$ ) =  $\mu_{NOO}$**

55  $= \hat{\mu}_{NOO} \frac{S_{NO_2}}{S_{NO_2} + K_{NO_2,NOO}} \frac{S_{O_2}}{S_{O_2} + K_{O_2,NOO}} \frac{S_{NHx}}{S_{NHx} + K_{NHx,ANO}} \frac{S_{PO_4}}{S_{PO_4} + K_{PO_4,ANO}} \frac{S_{ALK}}{S_{ALK} + K_{ALK,NOO}}$

56  $- \hat{b}_{NOO,O_2} \frac{S_{O_2}}{S_{O_2} + K_{O_2,NOO}} - \hat{b}_{AOO,NOx} \frac{S_{NO_3} + S_{NO_2}}{S_{NO_3} + S_{NO_2} + K_{NOx,ANO}} \frac{K_{O_2,NOO}}{S_{O_2} + K_{O_2,NOO}}$

57  $- \hat{b}_{NOO,ANA} \frac{K_{NOx,ANO}}{S_{NO_3} + S_{NO_2} + K_{NOx,ANO}} \frac{K_{O_2,NOO}}{S_{O_2} + K_{O_2,NOO}}$

58 Where (in addition to above):

59  $\hat{\mu}_{NOO} = \text{maximum specific growth rate of NOO} (d^{-1}) = 0.7$

60  $K_{NO_2,NOO} = NOO \text{ half saturation coefficient for } (NO_2^-) \left( \frac{mgN}{L} \right) = 0.1$

61  $K_{O_2,NOO} = NOO \text{ half saturation coefficient for dissolved } O_2 \left( \frac{mgO_2}{L} \right) = 0.1$

62  $K_{NHx,ANO} = \text{Nitrifier nutrient half saturation coefficient for } (NH_4^+$

63  $+ NH_3) \left( \frac{mgN}{L} \right) = 0.001$

64  $K_{ALK,NOO} = NOO \text{ half saturation coefficient for alkalinity} \left( \frac{meq}{L} \right) = 0.5$

65  $\hat{b}_{NOO,O_2} = \text{maximum specific aerobic decay rate of NOO} (d^{-1}) = 0.15$

66  $\hat{b}_{NOO,NOx} = \text{maximum specific anoxic decay rate of NOO} (d^{-1}) = 0.07$

67  $\hat{b}_{NOO,ANA} = \text{maximum specific anaerobic decay rate of NOO (d}^{-1}\text{)} = 0.04$

68

69

70 **AOO and NOO washout SRT calculation**

71

72 The modeled SRT to avoid washout for NOO was calculated by taking the inverse of

73 average modeled  $\mu_{NOO}$  values (as shown above, calculated approximately every minute) over

74 one cycle, i.e.:

75 
$$\text{washout } SRT_{NOO} = \frac{1}{\text{mean}(\mu_{NOO})}$$

76 A similar calculation was done for AOO to affirm that modeled SRT was sufficiently

77 high to retain AOO. The aerobic fraction of the resulting SRT for AOO and NOO was then

78 calculated by assuming that 48% of the intermittently aerated react phase was aerobic – see

79 Section 2.1 for details.

80 
$$SRT_{AER} = SRT * \frac{0.48(t_{AER})}{t_{AN} + t_{AER}} = SRT * 0.399$$

81 Where:

82  $SRT_{AER} = \text{aerobic SRT}$

83  $t_{AER} = \text{length of modeled intermittently aerated react phase (minutes)}$

84  $= 222 \text{ (variable in the actual reactor)}$

85  $t_{AN} = \text{length of anaerobic react phase (minutes)}$

86  $= 45 \text{ (same in the actual reactor)}$

87

88 **specific growth rate of PAOs on PHA and O<sub>2</sub> (d<sup>-1</sup>) =  $\mu_{PAO,O_2}$**

89 
$$= \hat{\mu}_{PAO} \frac{\frac{X_{PHA}}{X_{PAO}}}{\frac{X_{PHA}}{X_{PAO}} + K_{PHA}} \frac{S_{O_2}}{S_{O_2} + K_{O_2,OHO}} \frac{S_{NHx}}{S_{NHx} + K_{NHx,OHO}} \frac{S_{PO}}{S_{PO} + K_{PO,PAO}} \frac{S_{ALK}}{S_{ALK} + K_{ALK}}$$

90

91 Where (in addition to above):

92  $\hat{\mu}_{PAO} = \text{maximum specific growth rate of PAOs (d}^{-1}\text{)} = 0.95$   
93  $X_{PHA} = \text{concentration of polyhydroxyalkanoates – PHAs } \left(\frac{\text{mgCOD}}{\text{L}}\right)$   
94  $X_{PAO} = \text{concentration of PAOs } \left(\frac{\text{mgCOD}}{\text{L}}\right)$   
95  $K_{PHA} = \text{half saturation coefficient for PHA } \left(\frac{\text{mgCOD}}{\text{L}}\right) = 0.1$   
96  $K_{O_2,OHO} = \text{OHO and PAO half saturation coefficient for}$   
97  $\text{dissolved } O_2 \left(\frac{\text{mgO}_2}{\text{L}}\right) = 0.05$   
98  $K_{NHx,OHO} = \text{OHO and PAO nutrient half saturation coefficient for (NH}_4^+$   
99  $\text{+ NH}_3) \left(\frac{\text{mgN}}{\text{L}}\right) = 0.001$   
100  $K_{PO_4,PAO} = \text{PAO half saturation coefficient for PO}_4^{3-} \left(\frac{\text{mgP}}{\text{L}}\right) = 0.15$   
101  $K_{ALK} = \text{PAO half saturation coefficient for alkalinity } \left(\frac{\text{meq}}{\text{L}}\right) = 0.1$

102  
103  
104 **specific growth rate of PAOs on PHA and  $NO_2^-$  ( $d^{-1}$ ) =  $\mu_{PAO,NO_2}$**   
105 
$$= \hat{\mu}_{PAO} \eta_{anox,PAO} \frac{\frac{X_{PHA}}{X_{PAO}}}{\frac{X_{PHA}}{X_{PAO}} + K_{PHA}} \frac{S_{NO}}{S_{NO_2} + K_{NO_2,OHO}} \frac{K_{O_2,OHO}}{S_{O_2} + K_{O_2,OHO}} \frac{S_{NHx}}{S_{NHx} + K_{NHx,OHO}}$$
  
106 
$$\frac{S_{PO}}{S_{PO_4} + K_{PO_4,PAO}} \frac{S_{ALK}}{S_{ALK} + K_{ALK}}$$

107  
108 Where (in addition to above):  
109  $\eta_{anox,PAO} = \text{PAO anoxic growth factor} = 0.33$   
110  $K_{NO_2,OHO} = \text{OHO and PAO half saturation coefficient for } NO_2^- \left(\frac{\text{mgN}}{\text{L}}\right) = 0.05$

111  
112  
113 **specific growth rate of PAOs on PHA and  $NO_3^-$  ( $d^{-1}$ ) =  $\mu_{PAO,NO_3}$**   
114 
$$= \hat{\mu}_{PAO} \eta_{anox,PAO} \frac{\frac{X_{PHA}}{X_{PAO}}}{\frac{X_{PHA}}{X_{PAO}} + K_{PHA}} \frac{S_{NO}}{S_{NO_3} + K_{NO_3,OHO}} \frac{K_{NO_2,OHO}}{S_{NO_2} + K_{NO_2,OHO}} \frac{K_{O_2,OHO}}{S_{O_2} + K_{O_2,OHO}}$$
  
115 
$$\frac{S_{NHx}}{S_{NHx} + K_{NHx,OHO}} \frac{S_{PO_4}}{S_{PO_4} + K_{PO_4,PAO}} \frac{S_{ALK}}{S_{ALK} + K_{ALK}}$$

116  
117  
118 Where (in addition to above):  
119  $K_{NO_3,OHO} = \text{OHO and PAO half saturation coefficient for } NO_3^- \left(\frac{\text{mgN}}{\text{L}}\right) = 0.1$   
120

### 121 S1.3. Solids Retention Time (SRT) Control

122 SRT was controlled via timed mixed liquor wasting after the aerated react period and  
123 before settling. A maximum wasting pump time was set on the PLC, and the actual pumping  
124 time for each cycle varied depending on the length of the aerated react phase. For example, if the  
125 maximum wasting pump time was set to 1 minute, the maximum aeration time was set to 300  
126 minutes, and the actual aeration time for a given cycle was 150 minutes, the actual pumping time  
127 would be  $1 \text{ minute} \times \frac{150 \text{ minutes}}{300 \text{ minutes}} = 0.5 \text{ minutes}$ . Because the aeration time varied on a cycle-  
128 by-cycle basis according to the influent strength and the target effluent  $\text{NH}_4^+$  level, the dynamic  
129 SRT value was calculated for each individual cycle, as adapted from Laurení et al. (2019) and  
130 Takács et al., (2008). SRT for each cycle was calculated according to the equation below  
131 (Laurení et al., 2019).

$$132 \quad SRT_{t+\Delta t} = SRT_t \left( 1 - \frac{X_E V_E + X_R V_W}{X_R V_R} \right) + \Delta t$$

133 Where:

134  $SRT_{t+\Delta t}$  = Solids retention time of cycle under analysis (days)

135  $SRT_t$  = Solids retention time of previous cycle (days)

136  $V_R$  = Volume of reactor (L)

137  $X_E$  = Effluent VSS concentration for the cycle under analysis (mg/L)

138  $V_E$  = Effluent volume for the cycle under analysis (L)

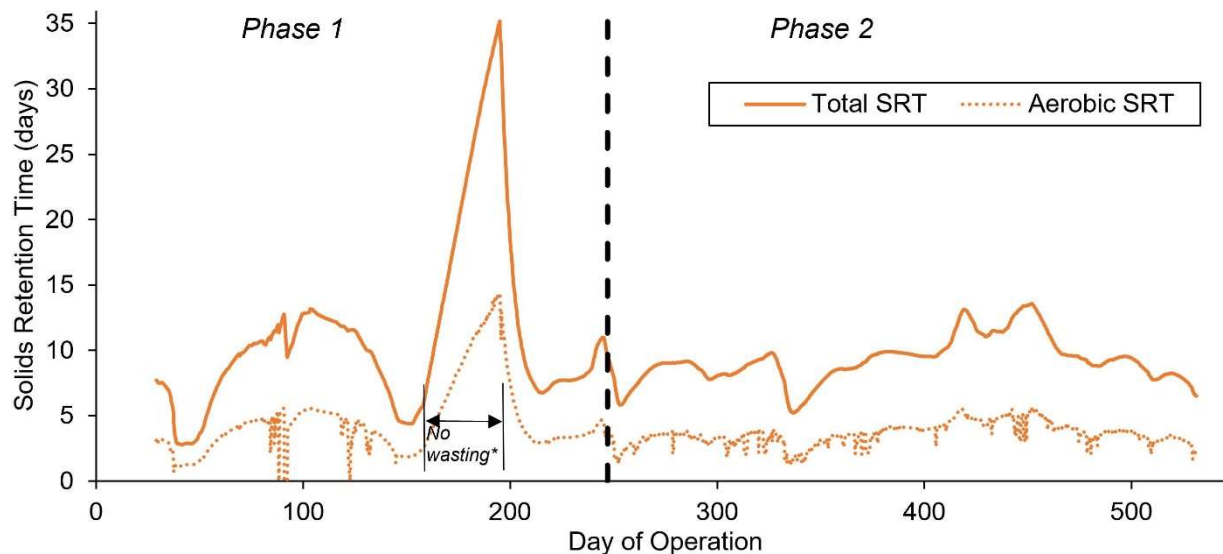
139  $X_R$  = Reactor MLVSS concentration for the cycle under analysis (mg/L)

140  $V_W$  = Mixed liquor wasting volume for the cycle under analysis (L)

141  $\Delta t$  = React time of the cycle under analysis, not including settling and decant (days)

142

143



144

145 **Figure S2.** Total and aerobic dynamic SRT over time in the SBR. The average total and aerobic  
 146 SRT during Phase 1 was  $11 \pm 7$  and  $4.5 \pm 3.0$  days, and the average total and aerobic SRT during  
 147 Phase 2 was  $9.2 \pm 1.8$  and  $3.6 \pm 0.9$  days, respectively. \*Mixed liquor wasting was suspended from  
 148 days 158 – 195 to recover AOO activity.

149

#### 150 **S1.4. 16S rRNA Gene Amplicon Sequencing**

151 16S rRNA gene amplicon library preparations were performed using a two-step PCR protocol  
 152 using the Fluidigm Biomark: Multiplex PCR Strategy as previously described (Griffin and Wells,  
 153 2017). In the first round of PCR, each 20  $\mu$ L reaction contained 10  $\mu$ L of FailSafe PCR 2X PreMix  
 154 F (Epicentre, Madison, WI), 0.63 units of Expand High Fidelity PCR Taq Enzyme (Sigma-Aldrich,  
 155 St. Louis, MO), 0.4  $\mu$ M of forward primer and reverse primer modified with Fluidigm common  
 156 sequences at the 5' end of each primer, 1  $\mu$ L of gDNA (approximately 100 ng) and the remaining  
 157 volume molecular biology grade water. The V4-V5 region of the 16S rRNA gene was amplified  
 158 in duplicate from 10 samples collected over the course of reactor operation using the 515F-Y (5'-  
 159 GTGYCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3') (Parada  
 160 et al., 2016) primer set. Thermocycling conditions for the 515F-Y/926R primer set were 95°C for  
 161 5 minutes, then 28 cycles of 95°C for 30 seconds, 50°C for 45 seconds, and 68°C for 30 seconds,



162 followed by a final extension of 68°C for 5 minutes. Specificity of amplification was checked for  
163 all samples via agarose gel electrophoresis.

164 Samples were then barcoded by sample via a second stage PCR amplification using Access  
165 Array Barcodes (Fluidigm, South San Francisco, CA) (Griffin and Wells, 2017). Each 20 uL PCR  
166 reaction consisted of 10 µL of FailSafe PCR 2X PreMix F, 0.63 units of Expand High Fidelity  
167 PCR Taq Enzyme, 2 µL of template from the first round of PCR, 4 µL of sample-specific barcode  
168 primers and the remaining volume molecular biology grade water. The conditions for the second  
169 round of PCR were 95°C for 5 minutes, then 8 cycles of 95°C for 30 seconds, 60°C for 30 seconds,  
170 and 68°C for 30 seconds. Agarose gel electrophoresis was run again after the second round of PCR  
171 to verify correct amplification. Sequencing was performed on an Illumina Miseq sequencer  
172 (Illumina, San Diego, CA) using Illumina V2 (2x250 paired end) chemistry.

173 For amplicon sequence analysis, sequence quality control was performed through DADA2  
174 (Callahan et al., 2016) integrated in QIIME2 version qiime2-2018.8 (Bolyen et al., 2018), which  
175 included quality-score-based sequence truncation, primer trimming, merging of paired-end reads,  
176 and removal of chimeras. Taxonomy was assigned to each individual sequence variation using the  
177 Silva database, release 132.

### 178 **S1.5 qPCR supermix and reaction conditions**

179 Bio-Rad iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) containing 50 U/ml iTaq  
180 DNA polymerase, 0.4 mM dNTPs, 100 mM KCl, 40 mM Tris-HCl, 6 mM MgCl<sub>2</sub>, 20 mM  
181 fluorescein, and stabilizers was used for two qPCR assays. Target genes included ammonia  
182 oxidizing bacterial *amoA* via the *amoA*-1F and *amoA*-2R primer set (Rotthauwe et al., 1997) and  
183 total bacterial (universal) 16S rRNA genes via the Eub519/Univ907 primer set (Burgmann et al.,  
184 2011). The final volume of the reaction mix for each PCR and qPCR reaction was 20 µl, in which

185 the DNA template was ~1 ng, and the primer concentrations were 0.2  $\mu$ M. All assays were  
 186 performed in triplicate. For each assay, triplicate standard series were generated by tenfold serial  
 187 dilutions ( $10^2$ - $10^8$  gene copies/ $\mu$ l).

## 188 **S2. Process Modeling Reproduces Key Elements of Process Performance**

189 Agreement between the process model and our experimental results suggest that the trends in  
 190 N and P removal from mainstream wastewater that we observed are likely generally applicable to  
 191 other locations. By closely modeling the influent (primary effluent), reactor control, aeration  
 192 control and SRT (model SRT 9.5 days, reactor SRT  $9.2 \pm 1.8$  days) from Phase 2, the resulting  
 193 model performance closely matched that of the reactor (Figure 5): modeled HRT was 7.2 hours  
 194 (reactor HRT  $6.8 \pm 2.8$  hours), modeled VSS was 1,245 mg/L (reactor VSS  $1,344 \pm 226$  mg/L)  
 195 and Figure 2, Figure 4, and Table 3 demonstrate that both in-cycle nutrient dynamics and effluent  
 196 concentrations were well-matched between the model and reactor performance. Importantly, this  
 197 was done via a commercially available wastewater process modeling software without  
 198 modification to the inCTRL ASM matrix.

199

## 200 **S3. Supporting Table and Figures**

201

202 **Table S1.** Influent (primary effluent) COD fractionation and COD-to-nutrient ratios.

	Primary Effluent	As percent of total COD
Total COD (mgCOD/L) <sup>a</sup>	164.4 $\pm$ 46.2	---
Particulate COD (mgCOD/L)	61.7 $\pm$ 23.8	37%
Colloidal COD (mgCOD/L)	28.6 $\pm$ 18.1	17%
Soluble COD not including VFA (mgCOD/L)	56.4 $\pm$ 19.4	34%
VFA (mgCOD/L)	18.8 $\pm$ 8.9	11%
COD:TP <sup>b</sup> (gCOD/gP)	67:1	---
COD:TKN <sup>b</sup> (gCOD/gN)	8.3:1	---

<sup>a</sup>Primary effluent COD fractionation was performed weekly from days 114 - 515 (n = 50).

<sup>b</sup>COD:Nutrient ratios are taken from average of all samples from days 27 - 519 (n = 192).

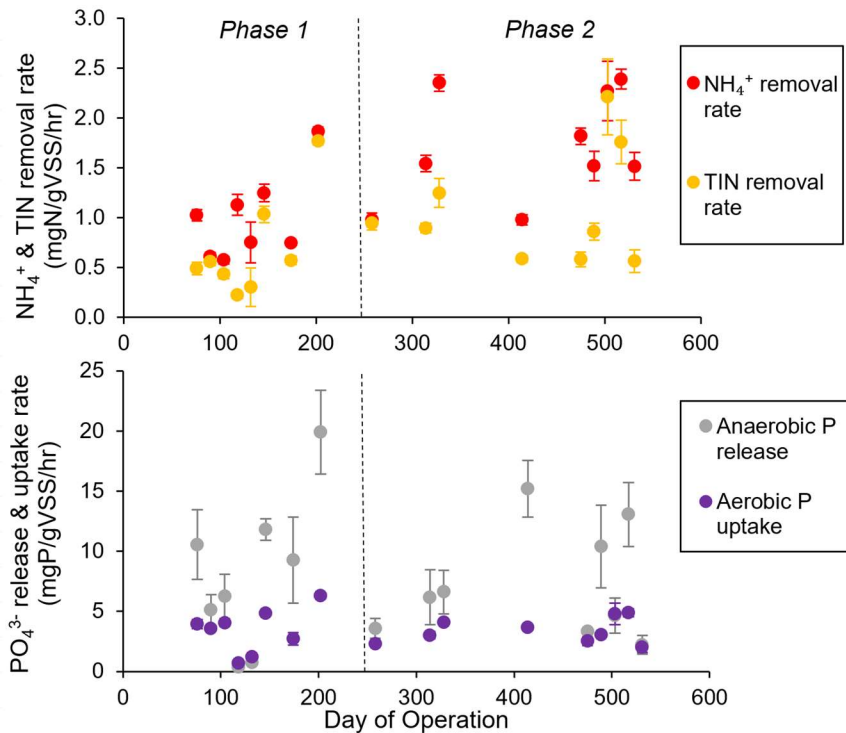
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205 **Table S2.** N<sub>2</sub>O emissions test results for 8 cycles during Phase 2.

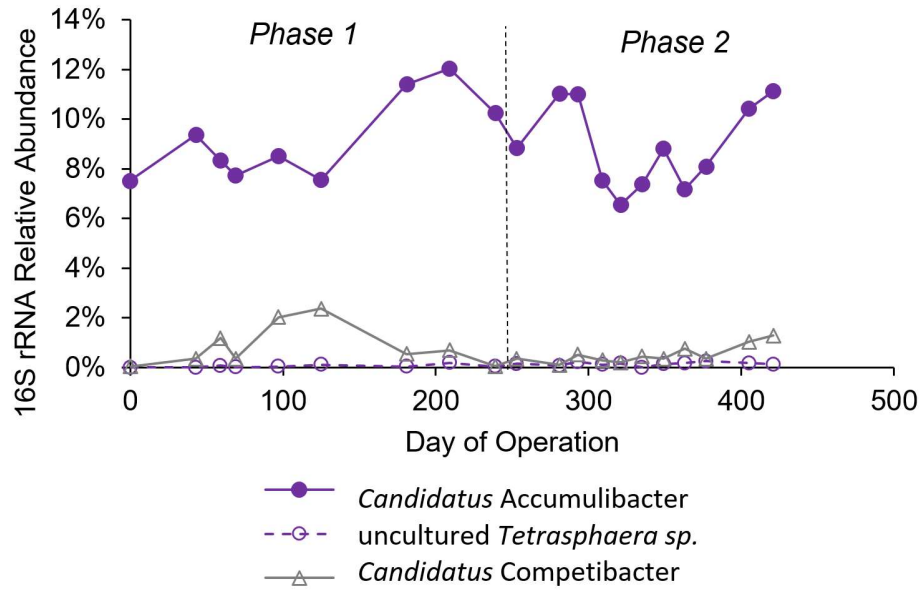
Day of cycle tested	N <sub>2</sub> O emitted/influent TKN	N <sub>2</sub> O emitted/ TIN removed	influent TKN (mgN/L)	influent COD (mg/L)	COD/ TKN	Effluent NO <sub>2</sub> <sup>-</sup> (mgN/L)	Average temp (°C)
414	3.8%	11.4%	23	206	9	2.9	20.5
426	6.2%	12.0%	20	204	10	2.7	20.3
428	1.0%	2.3%	12	140	12	1.2	20.5
475	1.0%	2.6%	13	64	5	2.0	20.4
489	2.2%	4.3%	19	183	10	2.4	20.3
503	0.2%	0.2%	14	160	11	0.4	19.4
517	0.8%	1.6%	21	147	7	1.9	19.4
531	1.56%	7.36%	13	144	11	2.1	19.4

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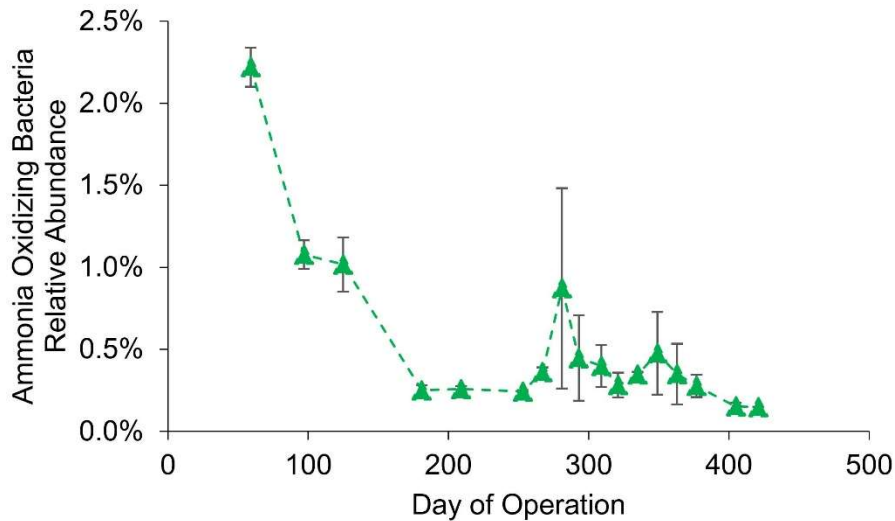
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**Figure S3.** In-cycle N and P removal rates from least-squares regression of the linear portions of in-cycle grab samples for NH<sub>4</sub><sup>+</sup>, NO<sub>2</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup>, and PO<sub>4</sub><sup>3-</sup>. Error bars represent standard errors of the slopes.



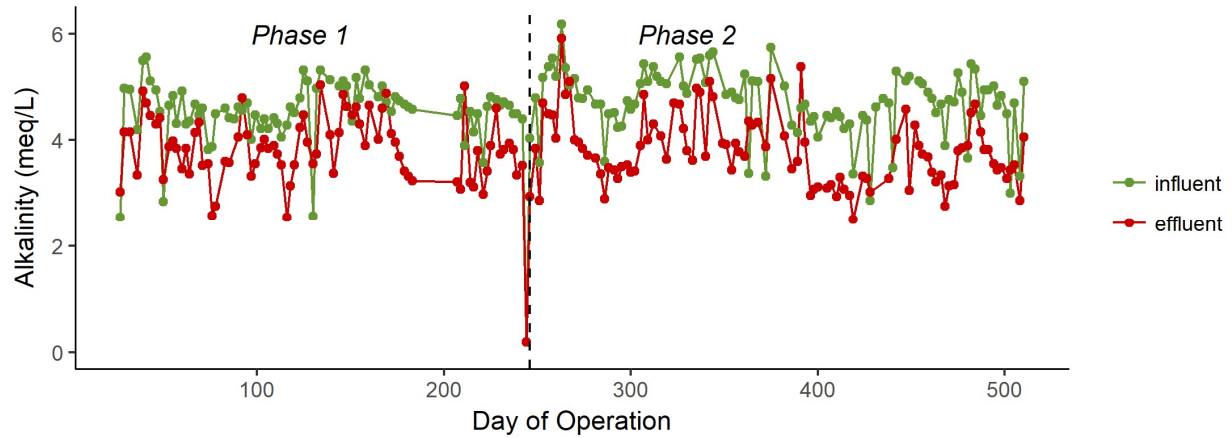
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 217 **Figure S4.** Relative *Accumulibacter*, *Tetrasphaera*, and *Competibacter* abundance through the  
 218 first 421 days of reactor operation according to 16S rRNA gene sequencing. Day “0” represents  
 219 the inoculum, which was sampled before reactor operation began.

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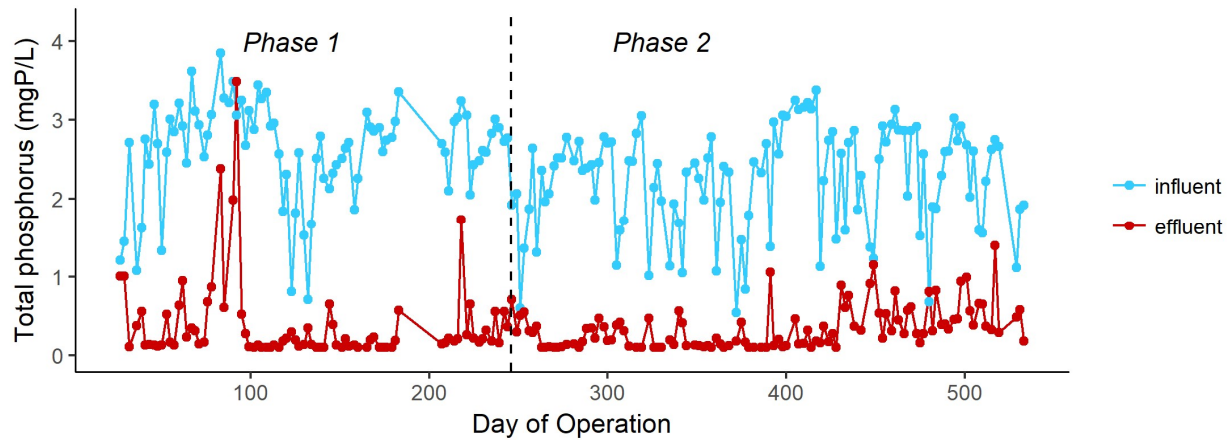
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 224 **Figure S5.** Ammonia oxidizing bacterial *amoA* gene abundance normalized to total bacterial 16S  
 225 rRNA genes through the first 421 days of reactor operation according to qPCR.

226  
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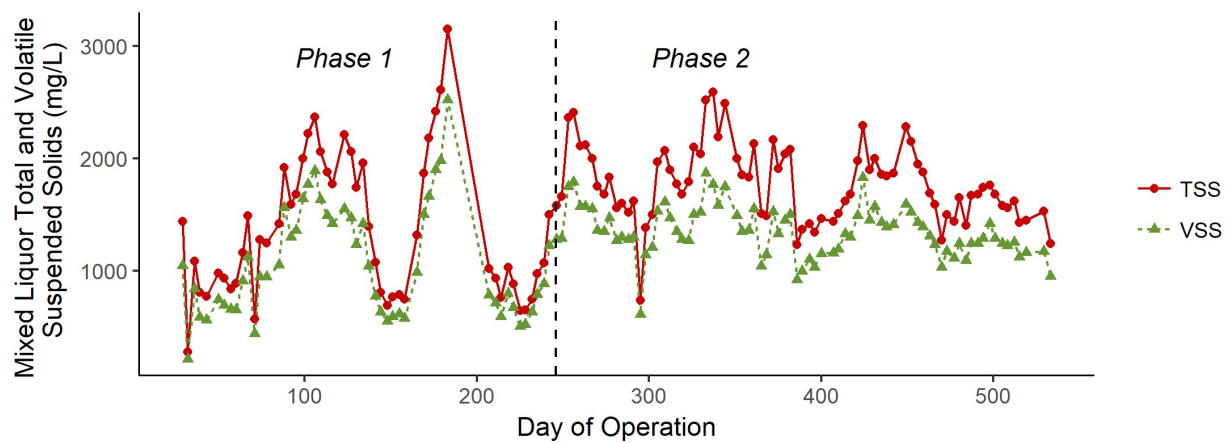
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229 **Figure S6.** Reactor influent and effluent alkalinity concentrations from composite sampling

230  
231



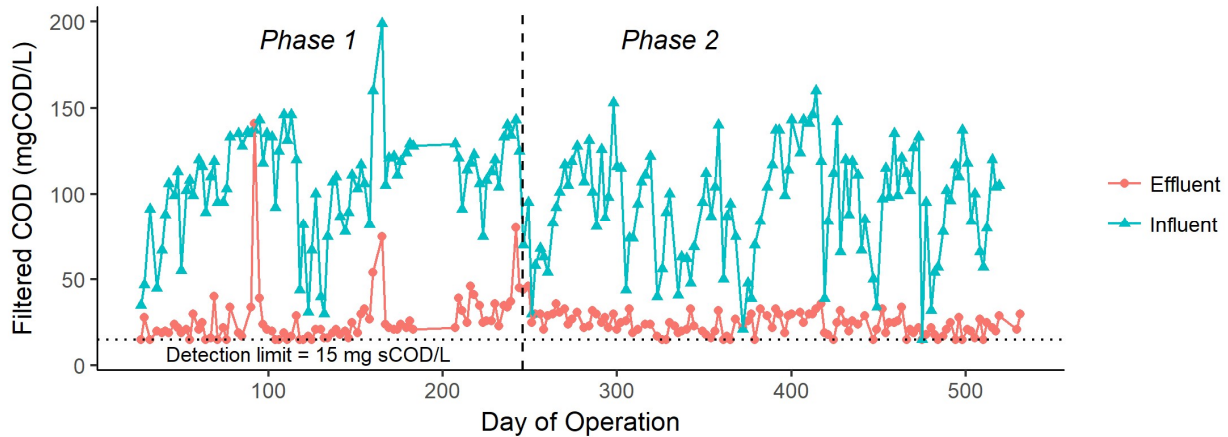
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233 **Figure S7.** Reactor influent and effluent total phosphorus concentrations from composite  
234 sampling.

235



236  
237 **Figure S8.** Reactor mixed liquor TSS and VSS concentrations.

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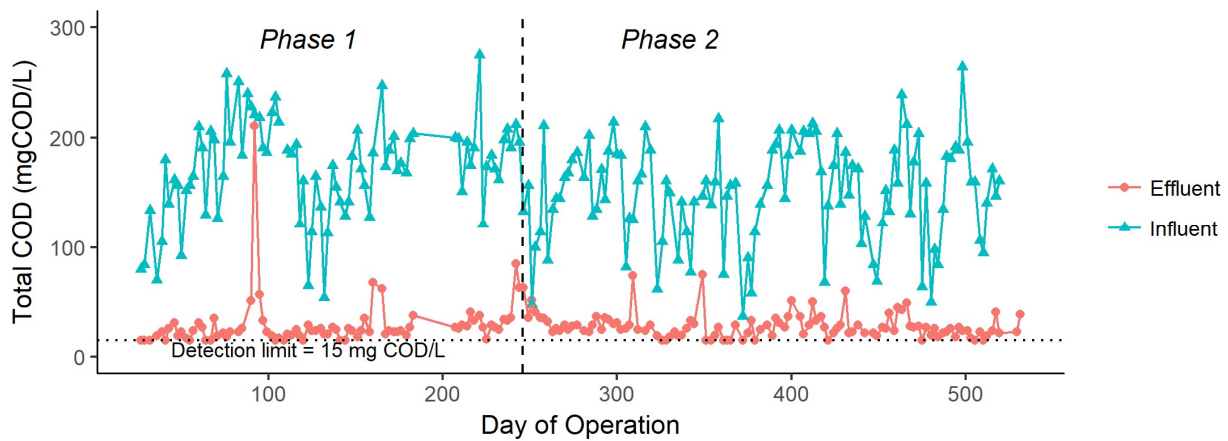
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**Figure S9.** Reactor influent and effluent filtered COD from composite sampling. Samples were filtered through a 1.2  $\mu\text{m}$  pore size membrane.

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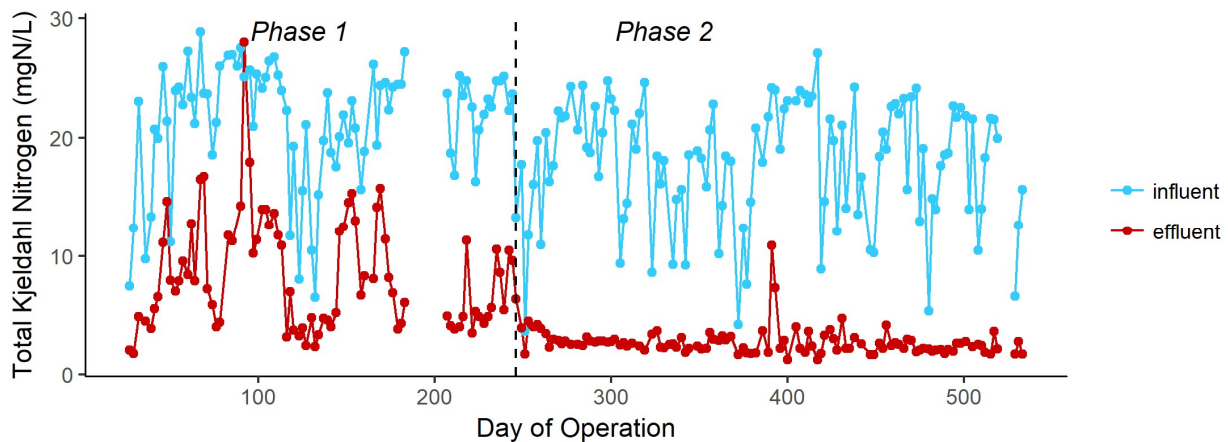


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244

**Figure S10.** Reactor influent and effluent total COD concentrations from composite sampling.

245



246

247

**Figure S11.** Reactor influent and effluent TKN concentrations from composite sampling.

249 **S3. References for Supporting Information**

250

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