

## Supplemental materials:

# Enhancing phosphorus removal of photogranules by incorporating polyphosphate accumulating organisms

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# Additions to materials and methods

## Bioreactor setup

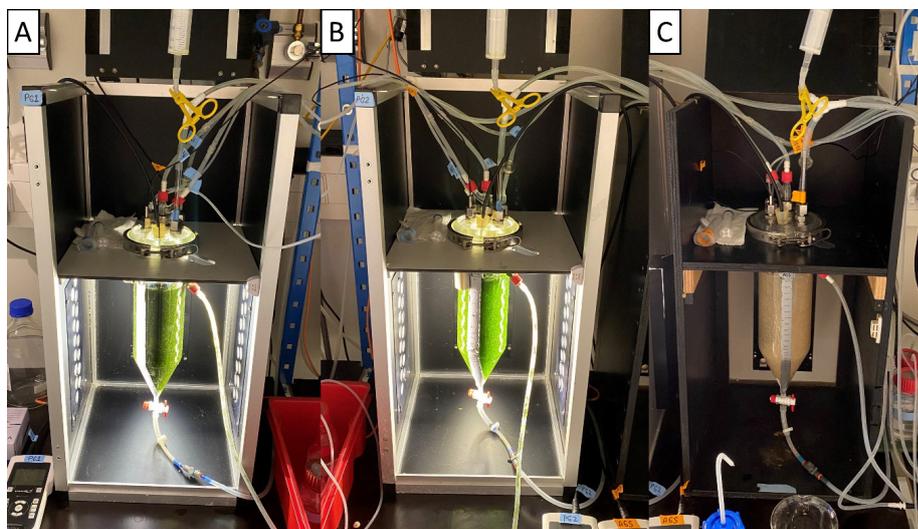


Figure S1: Bubble column bioreactor setup for A) PG1, B) PG2 and C) AGS.

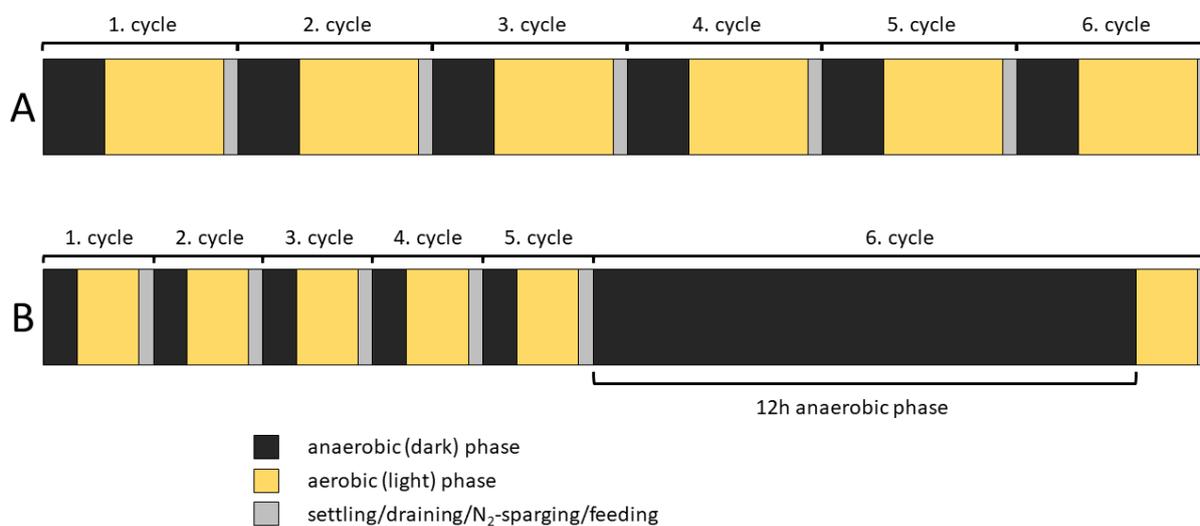
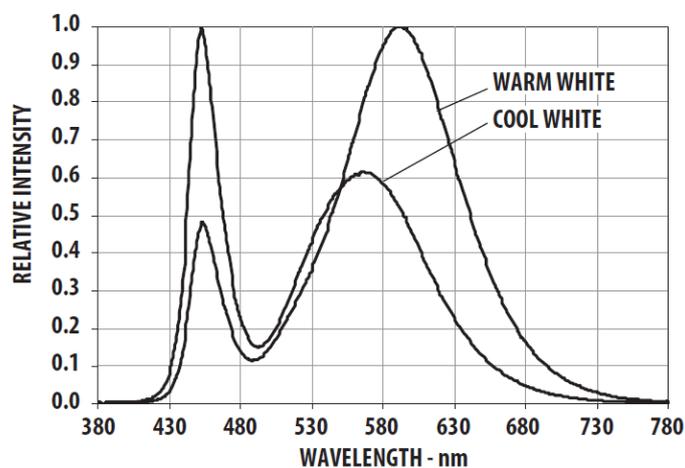


Figure S2. Sequencing batch cycles during 24 hours. A) Six equal cycles of anaerobic phase (70 min), aerobic reaction (150min), settling (5min), effluent removal (3min), nitrogen sparging (7min), and feeding (5min). B) Five cycles with equal anaerobic phases of 40 min and aerobic phases of 80 min. One cycle had an extended anaerobic phase of 12 hours (720min) while maintaining an aerobic phase of 80 min. All cycles had an equal phase for settling (5min), effluent removal (3min), nitrogen sparging (7min), and feeding (5min).

### Information on LED light spectrum



**Figure S3: Light spectrum of LED lamps** represented as relative intensity vs. wavelength as provided by the manufacturer (Avago Technology, USA). The warm white LED lamps were used for the experiment.

### Analytical methods

Acetate was measured as acetic acid by an Agilent 1200 series high-performance liquid chromatography (HPLC) with a Hi-Plex H anion exchange column, with a particle size of 8  $\mu\text{m}$ , a length of 300 mm and an inner diameter of 7.7 mm (Agilent Technologies, Santa Clara, US). The method was adapted from the application note of the column (Ball and Lloyd, 2011) and other studies using a similar HPLC and column setup (D'Hondt et al., 2020; De Sitter et al., 2018; Kaur and Elst, 2014). Specifically, the column temperature was set to 50  $^{\circ}\text{C}$ , the eluent was 100% 0.01 M Sulfuric acid, and the flow was 0.6 mL/min. A UV detector was used to detect acetic acid and was set to 210 nm.

### *Chlorophyll-a and chlorophyll-b extraction and determination*

Chlorophyll was extracted with ethanol (100%) from biomass samples from PG1 and PG2 according to the protocol of Cuaresma Franco et al. (2012). Per analysis, three times 5 mL of mixed liquor from the end of the aerobic phase was transferred into a 15 mL light-blocking centrifuge tube (VWR) and subsequently centrifuged at 4696 rcf for 10 minutes. The supernatant was discarded, and the pellet was stored in a freezer at  $-18^{\circ}\text{C}$  until the extraction was performed. The full extraction protocol can be found in the supplemental materials. To improve the extraction of chlorophyll from the pellet, a metal ball (1mm) was added to each tube to allow for additional disruption of the pellet during vortexing and sonication steps. After the chlorophyll was extracted from the pellets, the concentrations of chlorophyll *a* and *b* were measured in spectrophotometrically using a quartz cuvette with a pathway of 1 cm. The absorbance at 652 nm and 665 nm was measured to quantify chlorophyll *a* and *b*. For optimal readings, the absorbance at 665 had to range between 0.2 and 0.8 and extracts were diluted accordingly. The final concentrations of chlorophyll *a* and *b* in the extracts were calculated using Equations 1-3, based on Lichtenthaler (1987).

#### Extraction protocol:

1. Transfer 5ml of biomass sample into a 15 mL light-blocking centrifuge tube
2. Centrifuge at 4400rpm at  $4^{\circ}\text{C}$  for 8 min
3. Discard supernatant and add 5ml of ethanol 100% and metal ball bearing to the pellet
4. Vortex vigorously and put centrifuge tube in an ultrasound bath for 5 min to disrupt the pellet
5. Incubate the biomass suspension at  $60^{\circ}\text{C}$  for 50min
6. Incubate the biomass suspension at  $4^{\circ}\text{C}$  for 15 min
7. Centrifuge the biomass suspension at 4400rpm at  $4^{\circ}\text{C}$  for 8 min
8. Add more ethanol if the pellet is not white after centrifugation and repeat the extraction
9. Measure chlorophyll content in a spectrophotometer at 652nm and 665nm in a quartz cuvette (blank done with ethanol)

Chlorophyll-a and chlorophyll-b were determined from absorbance at 652nm and 665nm according to the Equations 1-3 after Lichtenthaler (1987):

$$Chl_a = (16.72 \cdot A_{665} - 9.16 \cdot A_{652}) \quad (1)$$

$$Chl_b = (34.09 \cdot A_{652} - 15.28 \cdot A_{665}) \quad (2)$$

$$Chl_{tot} = Chl_a + Chl_b \quad (3)$$

With  $Chl_a$  = concentration of chlorophyll *a* in  $\mu\text{g mL}^{-1}$

$Chl_b$  = concentration of chlorophyll *b* in  $\mu\text{g mL}^{-1}$

$A_{665}$  = absorbance at 665 nm

$A_{652}$  = absorbance at 652 nm

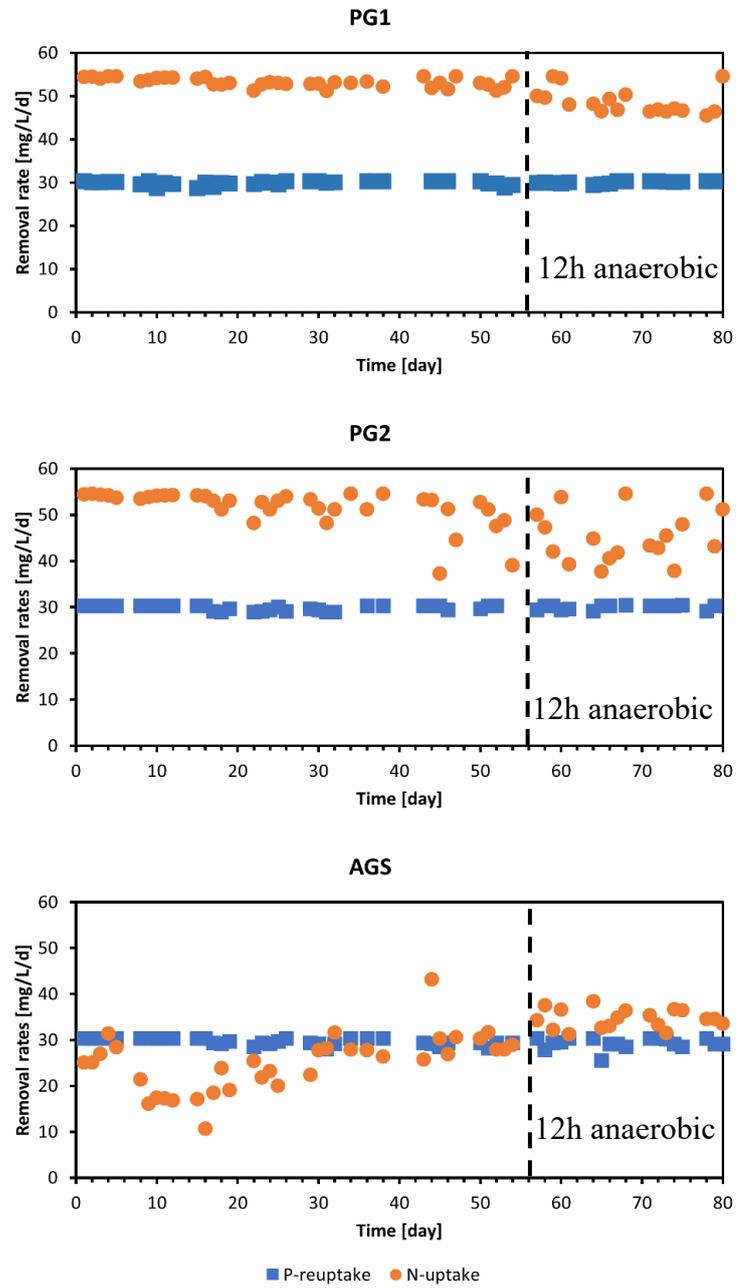
### *Microbial community assessment*

The microbial community of day 0, 8, 15, 31, 53, 67, and 79 was assessed using 16S and 18S rRNA gene amplicon sequencing according to Trebuch et al. (2020). Specifically, 15 mL of harvested photogranules were homogenised by a glass/Teflon tissue grinder, aliquoted into five 2 mL Eppendorf tubes, centrifuged at 14,870 rcf for 10 min and the supernatant discarded. The cell pellets were immediately frozen at -80 °C until further processing. DNA of 200 mg of wet cell pellet was extracted in triplicate by using the DNeasy PowerSoil Pro Isolation Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The quantity and quality of DNA were spectrophotometrically determined with a NanoDrop One (ThermoFisher Scientific, USA). The DNA samples were submitted for sequencing to Génome Québec (MacGill University, Montreal, CA). The 16S rRNA gene V3/V4 variable region was amplified using primer pair 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) (Herlemann et al., 2011). The 18S rRNA gene V4 variable region was amplified using the primer pair 616\*F (TTAAARVGYTCGTAGTYG) and 1132R (CCGTCAATTHCTTYAART) (Hugerth et al., 2014). Both sets of primers were

modified to add Illumina adapter overhang nucleotide sequences to the gene-specific sequences. Sequencing was performed using an Illumina MiSeq system (Illumina MiSeq, USA) with 300-bp reads (v3 chemistry). The obtained sequences were processed with DADA2 (Callahan et al., 2016). Taxonomic alignment of the sequences was done against the SILVA database (release 138) using SINA (<https://www.arb-silva.de>). The 16S and 18S dataset was normalized using the cumulative sums scaling (CSS) function of the R package *metagenomSeq* version 1.24.1 (Paulson et al., 2013). The analysis of the microbiome data was performed with the R-package *phyloseq* (version 1.26.1) (McMurdie and Holmes, 2013) and *ampvis2* (version 2.7.4) (Andersen et al., 2018). The community structure and the change through time of the 16S and 18S dataset were analysed by Principal Coordinate Analysis (PCoA) using weighted UniFrac distance (Lozupone et al., 2011).

## Additions to results

*Nutrient removal, biomass concentration, sludge volume index and nutrient load*



**Figure S4:** Nitrogen and phosphorus removal rates of PG1, PG2 and AGS.

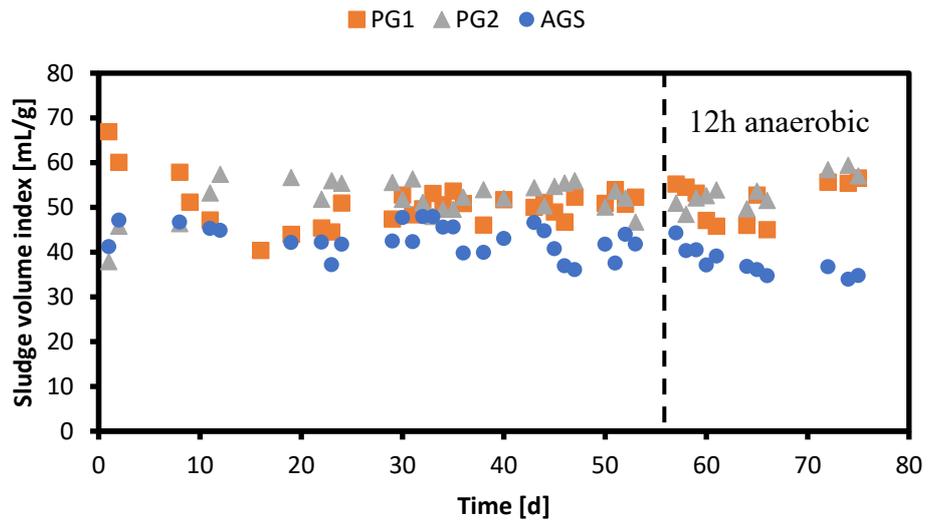
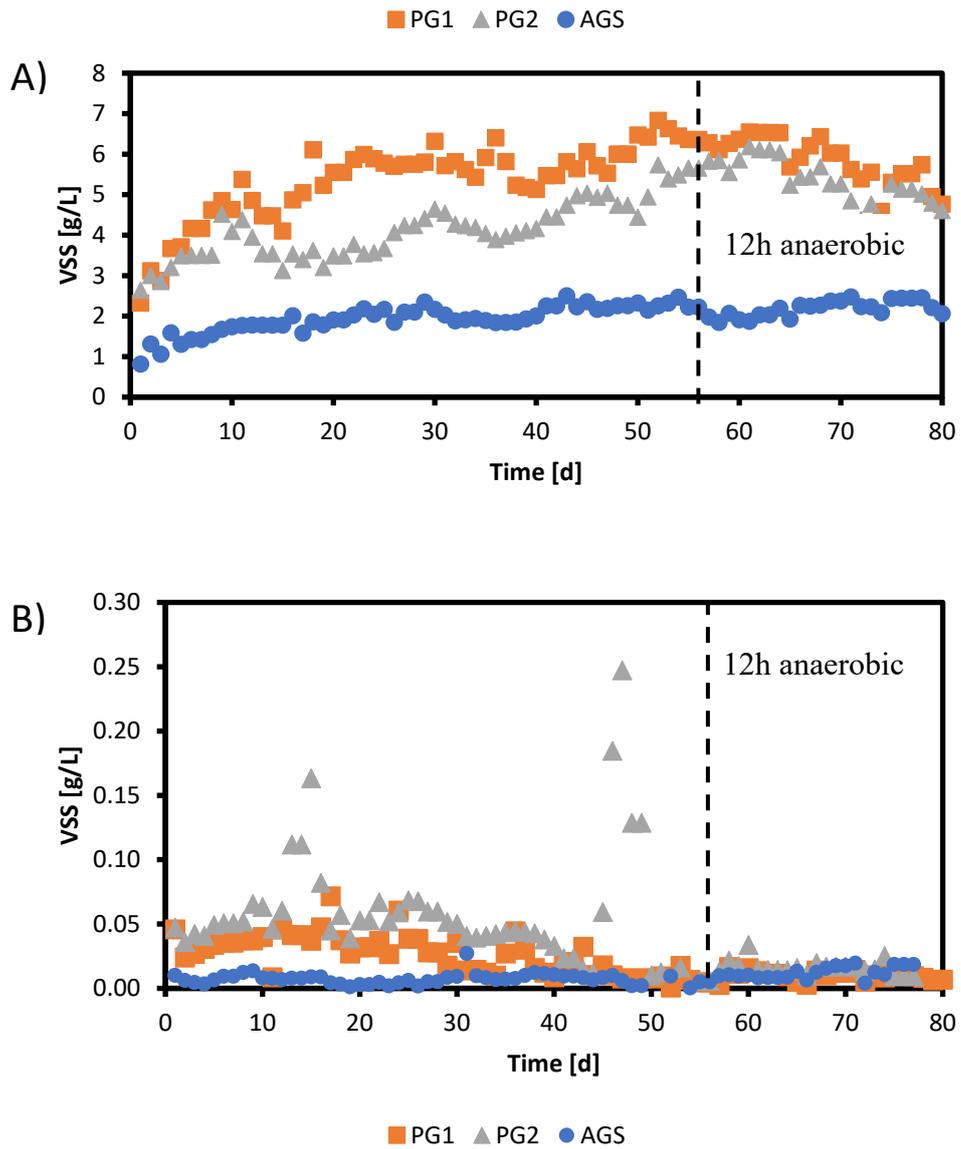


Figure S5: Sludge volume index of the bioreactor systems.



**Figure S6: Biomass concentration** represented as TSS and VSS in the bioreactor systems (A) and effluent (B) over time.

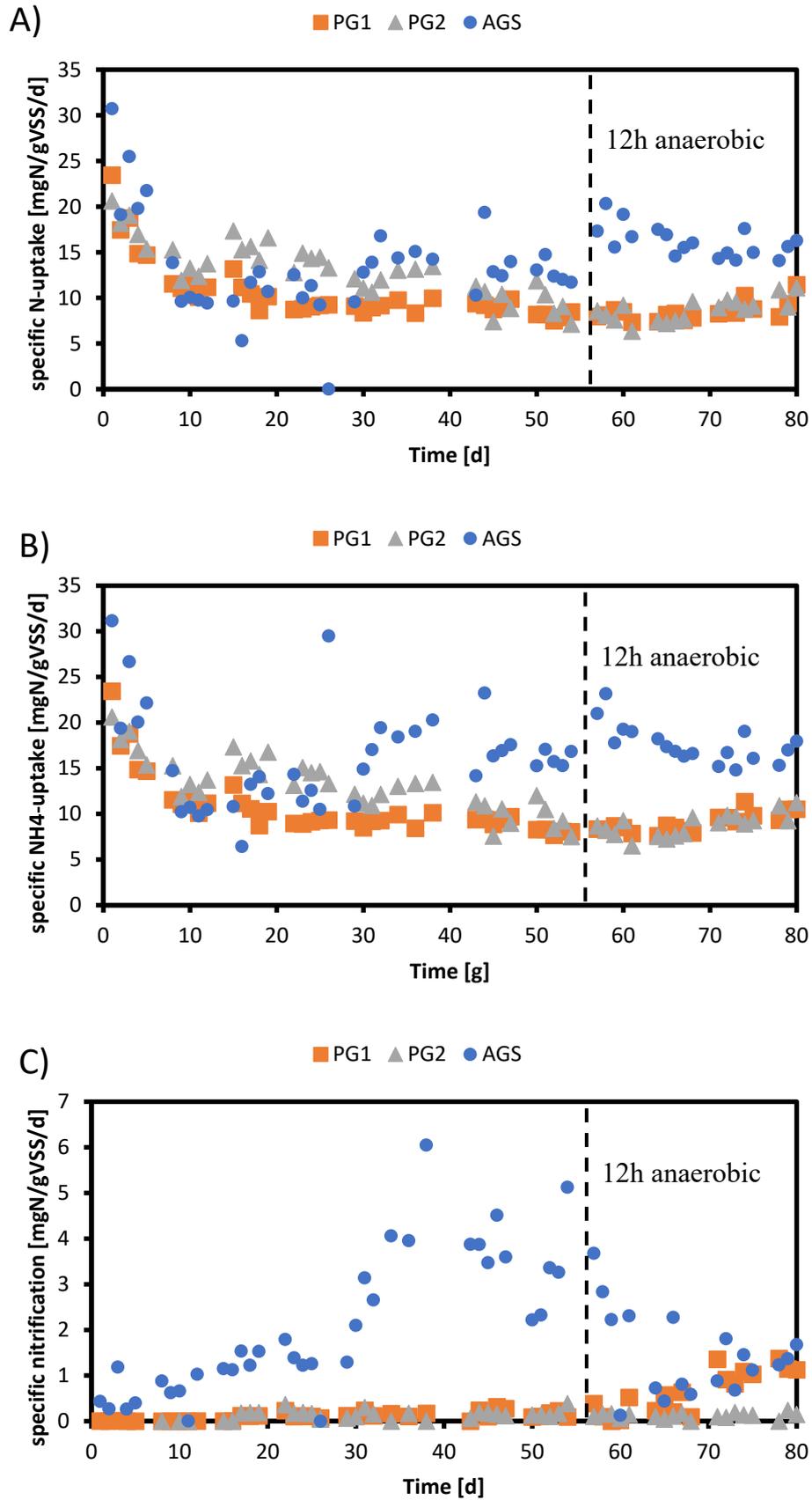


Figure S7: A) Specific N-uptake over time. B) Specific NH<sub>4</sub>-uptake over time. C) Specific nitrification over time.

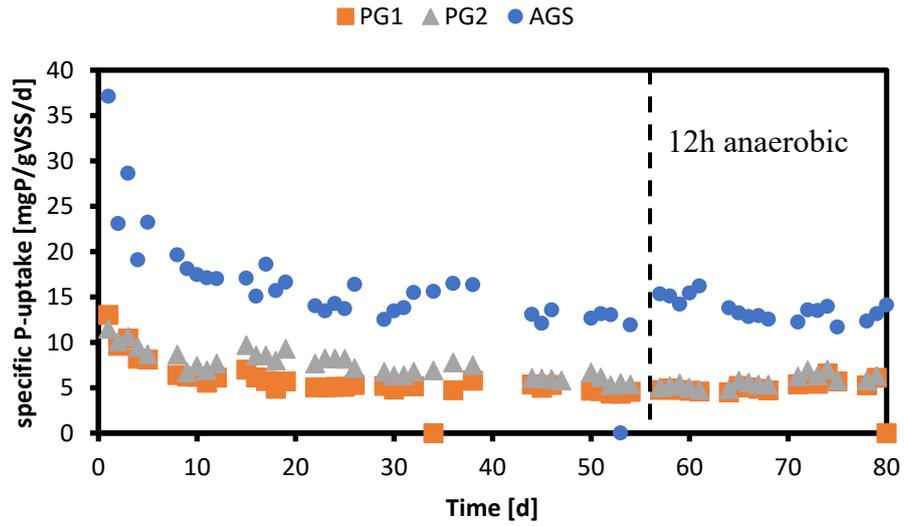


Figure S8: Specific P-uptake over time.

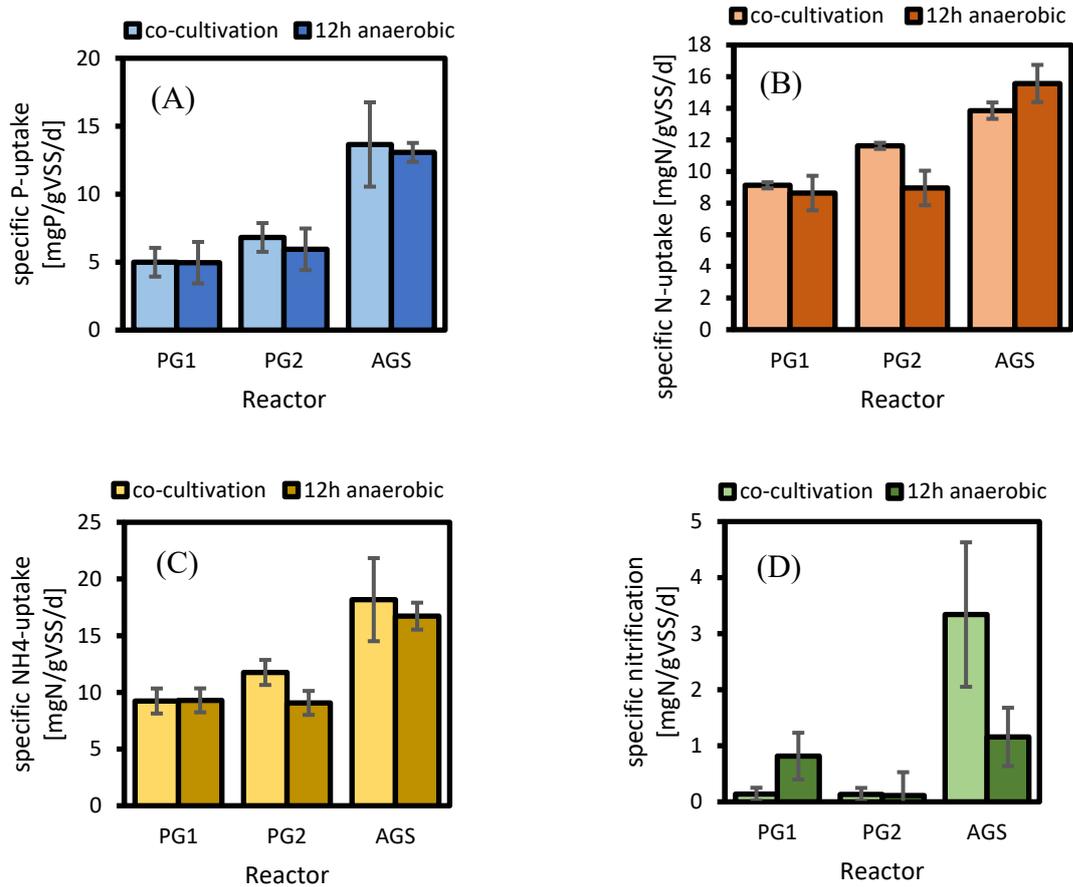
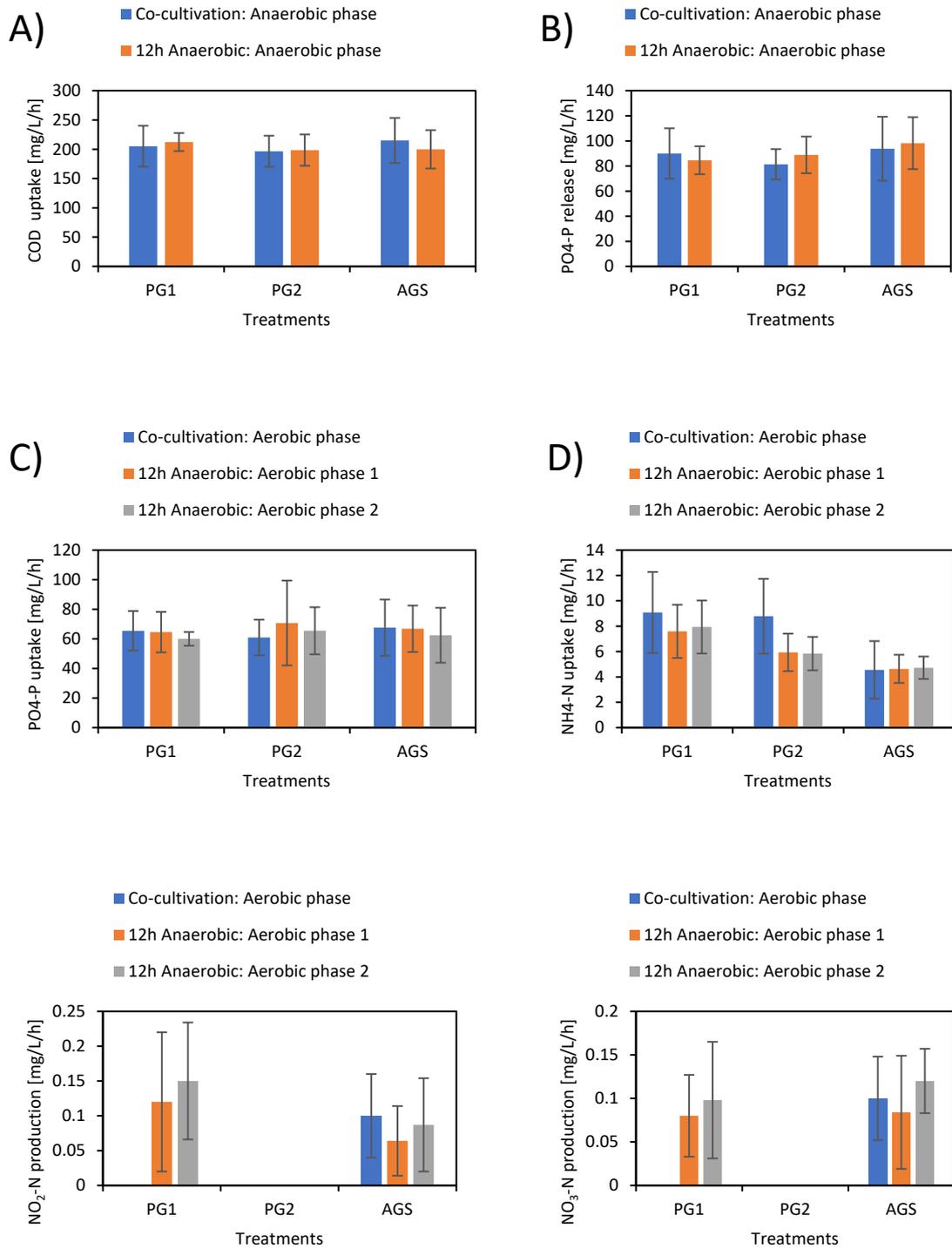


Figure S9: Average specific P-uptake (A), N-uptake (B), NH<sub>4</sub>-uptake (C) and nitrification (D) from the co-cultivation phase (day 10-56) and 12h anaerobic phase (day 56-80). The error bars represent the standard deviation.



**Figure S10: Specific removal rates observed during one cycle (on day 4, 11, 15, 25, 30, 36, 46, and 53) or two consecutive cycles (on day 67, 73, and 79) in 5- or 10-min intervals. A) The average COD uptake rate during the anaerobic phase. B) The average P release rate during the anaerobic phase. C) The average PO<sub>4</sub>-P uptake rate during the aerobic phase. D) The average NH<sub>4</sub>-N uptake rate during the aerobic phase. E) The average NO<sub>2</sub>-N production during the aerobic phase. E) The average NO<sub>3</sub>-N during the aerobic phase. During the operation with a 12h anaerobic phase, two aerobic phases were monitored: here denoted as Aerobic phase 1 and Aerobic phase 2.**

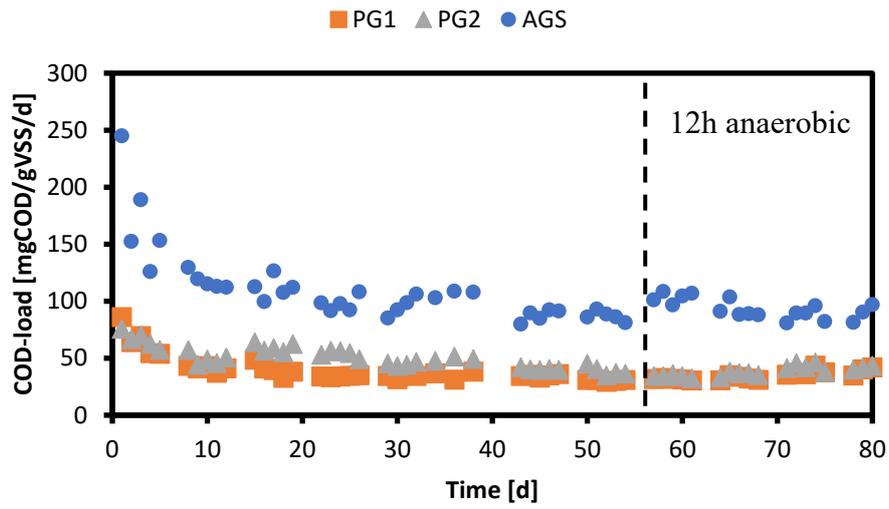


Figure S11: COD-load over time

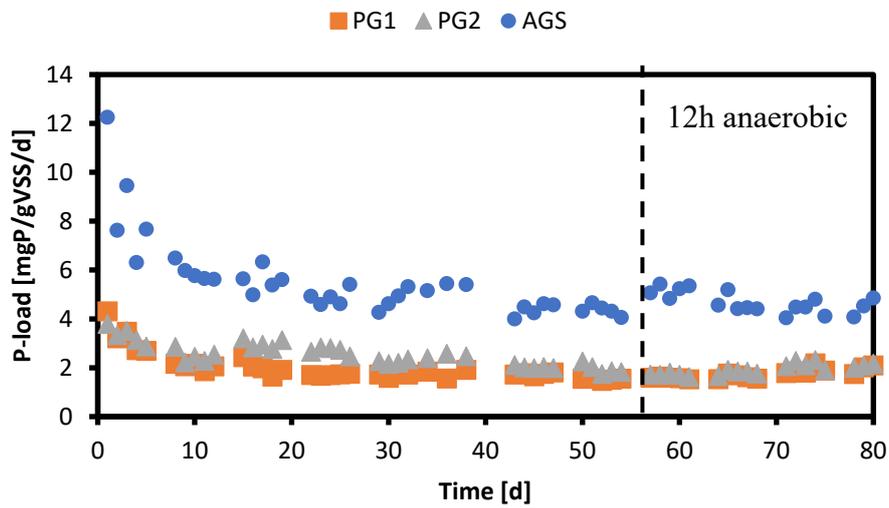
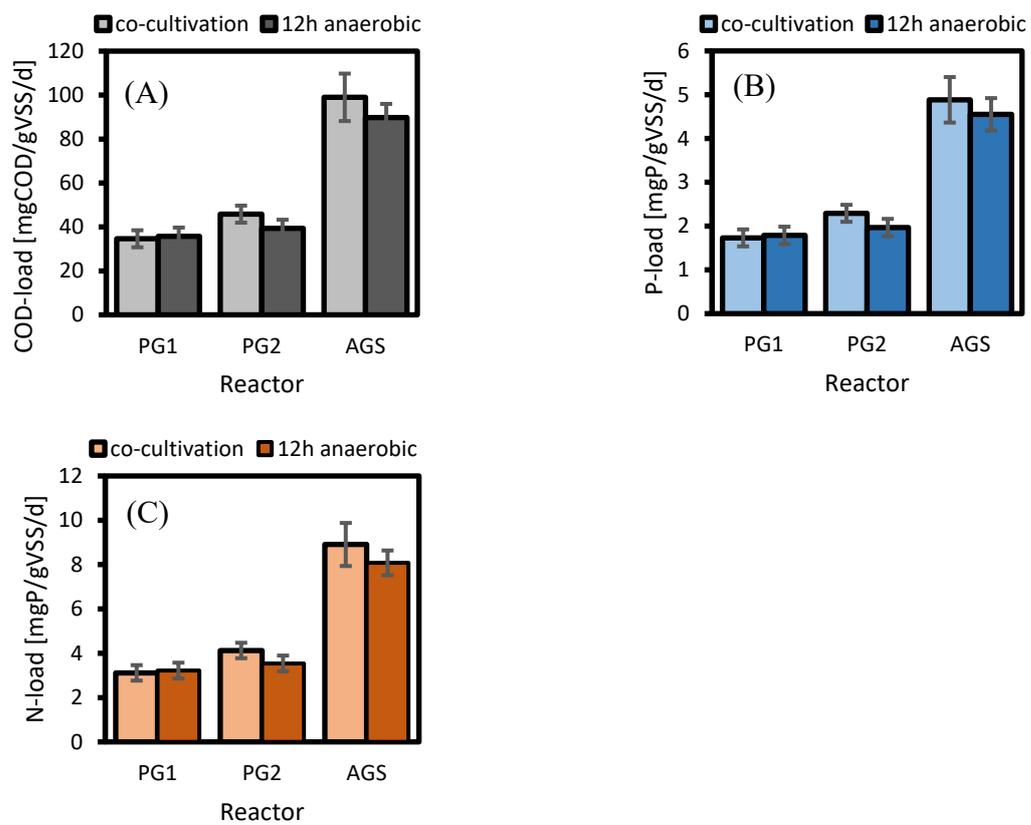


Figure S12: P-load over time.



**Figure S13:** Averaged COD-load (A), P-load (B) and N-load (C) from the co-cultivation phase (day 10-56) and 12h anaerobic phase (day 56-80). The error bars represent the standard deviation.

## Microbial community analysis

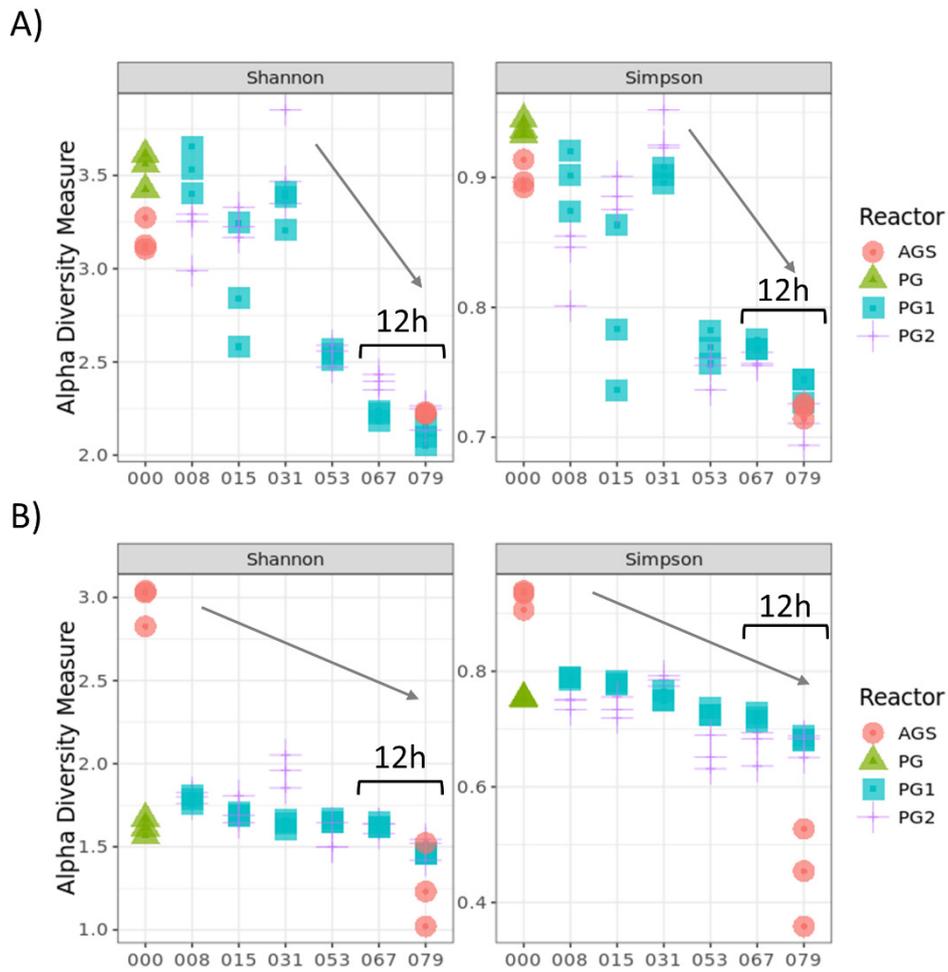
**A)**

	AGS		PG	PG1						PG2					
Candidatus_Accumulibacter	30.5	52.1	0	31.4	48.4	28.3	5.6	11.6	5.5	46.8	35.7	26.2	11.1	16.6	9.8
Alkalinema_CENA528	0	0	2.7	2.4	3.7	11.9	47.6	42.7	45.4	1.6	2.3	6.2	49.2	47.3	52.7
Prostheco bacter	2.1	9.3	5.3	8.7	5.4	9.9	5.5	2.2	2.9	6.4	7.1	12.6	6.4	3.6	1.7
Mesorhizobium	8.5	0.7	0	7.9	9.7	8.4	3.4	1.8	1.3	6	15.4	10.4	6.2	4.8	3.3
Candidatus_Competibacter	0.1	0	0	0	0	0.2	6.6	22	26.2	0	0.1	0.2	3.2	8.2	12.6
f_Saprosiraceae_ASV1079	0	0.2	0.2	2.9	3.3	2.8	3.7	2.4	1	3.1	6.3	6.5	3	2.3	1.4
Methylorosula	14.8	12	0	0.6	0.3	0.9	0.9	1.1	2.5	0.7	0.3	0.5	0.5	1	2.3
Leptolyngbya_PCC-6306	0	0	4.7	3.8	3.2	3.5	3.2	2.1	1.9	1.7	2.3	2.3	1.3	0.8	0.6
f_Phormidiaceae_ASV941	0	0	17.1	4.5	1.8	0.6	0.2	0	0	2.7	0.8	0.6	0.1	0.2	0
f_Chloroflexaceae_ASV868	0	0	0.2	0.8	1.7	4.8	5.5	3.7	3	0.4	1	1.2	0.9	1.1	1.6
f_env.OPS_17_ASV425	12.2	0.1	0	4.2	1.5	0.5	0.1	0	0	3.6	2.1	0.8	0.1	0.1	0
Xanthobacter	0.4	0	14.9	3.5	0.8	0.3	0	0	0	1.6	0.5	0.2	0	0	0
f_Chloroflexaceae_ASV322	0	0	0	0.5	1	3	2.9	2.1	1.7	0.1	0.5	0.8	0.5	0.7	0.9
Gemmobacter	1	0.8	0	0.6	0.7	1.6	0.7	0.3	0.3	1	1.6	3.1	1.3	0.6	0.5
Runella	0.2	0.2	0.4	0.2	0.2	1.6	1.4	1	1.2	0.3	0.2	1.1	1.7	1.7	2.2
Shinella	0.4	0.5	7.3	1.4	0.7	0.6	0	0	0	1	0.7	0.7	0.2	0	0
Hydrogenophaga	2.4	0.2	6.9	0.8	0.2	0.2	0.1	0.1	0.1	0.3	0.4	0.3	0	0	0.1
Flavobacterium	1.4	4.2	0	0.2	0.6	0.5	0	0	0.1	0.5	1.5	0.9	0.1	0	0
Aminobacter	1.1	5	0	0.4	0.4	0.3	0	0	0.1	0.4	0.7	0.4	0.3	0.2	0.2
Defluviicoccus	0.1	0	0	0.1	0.1	0.2	0.4	0.4	0.4	0.1	0.1	0.3	1.3	2.6	3.3
Remaining taxa (522)	24.8	14.9	40.2	25	16.3	19.9	12	6.5	6.3	21.6	20.5	24.6	12.6	8.2	6.8
	000	079	000	008	015	031	053	067	079	008	015	031	053	067	079

**B)**

	AGS		PG	PG1						PG2					
Chlorella	0.3	0	60.2	51.8	38.6	32.6	29.3	40.3	44.4	58.1	54.6	43.8	23.3	25.4	37.3
Desmodesmus	0	0	9.5	18.1	15.1	19	46.2	45.4	47.7	10.4	11.9	22.3	54.9	53.6	50.5
p_Cryptomycota_ASV72	6.8	2.3	0	7.4	32.1	38.3	9.9	4.3	1.6	2.5	8.5	11.3	10.5	7	1
Botryosphaerella	0.2	0	27	19.8	12.5	8.1	2.7	1.6	1	22.2	20.7	13.4	5.7	2.9	2.1
Rhogostoma	1	74.1	0	0.5	0.2	0.5	10.2	4	0.9	0.2	0.5	0.6	2.1	4.1	2
Geotrichum	28	5.1	0	0.3	0.1	0	0	0.1	0.1	0.2	0.1	0	0	0	0
Paraphysoderma	0	0	0	0	0	0	0.5	3.3	3.6	0	0	0	0.3	4.1	5.8
Vermamoeba	8.1	5.5	0.5	0.3	0.2	0.2	0	0	0	0.4	0.2	0.6	0.4	0.2	0.1
k_Eukaryota_ASV189	5.1	0.4	0.1	0.2	0.1	0.1	0	0	0	0.4	0.3	0.7	0.2	0.2	0.1
k_Eukaryota_ASV93	5	0.4	0.1	0.2	0.1	0.1	0	0	0	0.5	0.3	0.7	0.2	0.2	0.1
k_Eukaryota_ASV51	4.5	0.3	0.1	0.2	0.1	0.1	0	0	0	0.4	0.3	0.7	0.2	0.1	0
Chlorococcum	0	0	0.9	0.8	0.4	0.3	0.1	0.1	0	1.4	1	1	0.5	0.2	0.1
k_Eukaryota_ASV197	3.6	0.3	0	0.1	0.1	0	0	0	0	0.3	0.1	0.3	0	0	0
k_Eukaryota_ASV166	3.2	0.2	0	0.1	0.1	0	0	0	0	0.2	0.2	0.5	0	0.1	0
Poteriespumella	0	4.3	0.2	0	0	0	0	0	0	0	0	0	0	0	0
k_Eukaryota_ASV19	2.6	0.4	0	0	0	0.1	0	0	0	0.3	0.2	0.5	0.1	0.1	0
k_Eukaryota_ASV263	3.5	0.2	0	0	0	0	0	0	0	0.1	0.1	0.3	0	0.2	0
Trichosporon	0	2.8	0	0	0	0	0.1	0.1	0.3	0	0	0.1	0.2	0.1	0.6
k_Eukaryota_ASV363	3.3	0	0.3	0	0	0	0	0	0	0.2	0.1	0.4	0.1	0	0
k_Eukaryota_ASV221	2.7	0	0.3	0	0	0	0	0	0	0.2	0.1	0.3	0.1	0	0
Remaining taxa (91)	22.5	3.6	0.8	0.3	0.2	0.6	1	0.9	0.4	1.7	0.9	2.4	1.2	1.4	0.4
	000	079	000	008	015	031	053	067	079	008	015	031	053	067	079

**Figure S14:** Microbial community composition of PG, PG1, PG2 and AGS assessed with 16S (A) and 18S (B) rRNA gene amplicon sequencing displayed from the inoculum (day 0), co-cultivation phase (day 8-53) and 12h anaerobic phase (day 67 and 79) at Genus level. The abundance is presented from the top 20 genera as the average of biological replicates. The remaining genera are summarized as “remaining taxa”.



**Figure S15:** Alpha diversity according to Shannon and Simpson of the microbial community of PG, PG1, PG2 and AGS assessed with 16S (A) and 18S (B) rRNA gene amplicon sequencing. The inoculum (day 0), co-cultivation phase (day 8-53) and 12h anaerobic phase (day 67 and 79) is displayed.

## References

- Andersen, K.S., Kirkegaard, R.H., Karst, S.M., Albertsen, M., 2018. ampvis2: an R package to analyse and visualise 16S rRNA amplicon data. *bioRxiv* 299537. <https://doi.org/10.1101/299537>
- Ball, S., Lloyd, L., 2011. Agilent Hi-Plex Columns for Carbohydrates , Alcohols , and Acids Organic Acids on Hi-Plex H. *Agil. Technol. Inc* 1–2.
- Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. <https://doi.org/10.1038/nmeth.3869>
- Cuaresma Franco, M., Buffing, M.F., Janssen, M., Vilchez Lobato, C., Wijffels, R.H., 2012. Performance of *Chlorella sorokiniana* under simulated extreme winter conditions. *J. Appl. Phycol.* 24, 693–699. <https://doi.org/10.1007/s10811-011-9687-y>
- D'Hondt, E., Soetemans, L., Bastiaens, L., Maesen, M., Jespers, V., Van den Bosch, B., Voorspoels, S., Elst, K., 2020. Simplified determination of the content and average degree of acetylation of chitin in crude black soldier fly larvae samples. *Carbohydr. Res.* 488, 107899. <https://doi.org/10.1016/j.carres.2019.107899>
- De Sitter, K., Garcia-Gonzalez, L., Matassa, C., Bertin, L., De Wever, H., 2018. The use of membrane based reactive extraction for the recovery of carboxylic acids from thin stillage. *Sep. Purif. Technol.* 206, 177–185. <https://doi.org/10.1016/j.seppur.2018.06.001>
- Herlemann, D.P.R., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J.J., Andersson, A.F., 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J.* 5, 1571–1579. <https://doi.org/10.1038/ismej.2011.41>
- Hugerth, L.W., Muller, E.E.L., Hu, Y.O.O., Lebrun, L.A.M., Roume, H., Lundin, D., Wilmes, P., Andersson, A.F., 2014. Systematic design of 18S rRNA gene primers for determining eukaryotic diversity in microbial consortia. *PLoS One* 9. <https://doi.org/10.1371/journal.pone.0095567>
- Kaur, G., Elst, K., 2014. Development of reactive extraction systems for itaconic acid: a step towards in situ product recovery for itaconic acid fermentation. *RSC Adv.* 4, 45029–45039. <https://doi.org/10.1039/C4RA06612J>
- Lichtenthaler, H.K., 1987. Chlorophylls and Carotenoids: Pigments of Photosynthetic Biomembranes. *Methods Enzymol.* 148, 350–382. [https://doi.org/10.1016/0076-6879\(87\)48036-1](https://doi.org/10.1016/0076-6879(87)48036-1)
- Lozupone, C., Lladser, M.E., Knights, D., Stombaugh, J., Knight, R., 2011. UniFrac: An effective distance metric for microbial community comparison. *ISME J.* 5, 169–172. <https://doi.org/10.1038/ismej.2010.133>
- McMurdie, P.J., Holmes, S., 2013. phyloseq: An R package for reproducible interactive analysis and

graphics of microbiome census data. PLoS One 8, e61217.  
<https://doi.org/10.1371/journal.pone.0061217>

Trebuch, L.M., Oyserman, B.O., Janssen, M., Wijffels, R.H., Vet, L.E.M., Fernandes, T.V., 2020. Impact of hydraulic retention time on community assembly and function of photogranules for wastewater treatment. *Water Res.* 173, 115506. <https://doi.org/10.1016/j.watres.2020.115506>