The Critical Role of Flocs in Nitrification in Full-Scale Aerobic Granular Sludge-Based WWTP

SUPPLEMENTARY INFORMATION

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S1 Calculations of substrate penetration and limiting compounds

According to Chen et al. (2020), the limiting compounds for a reaction in a biofilm can be calculated as follows:

$$\frac{S_{LF,e.d.}}{S_{LF,e.a.}} < \frac{1}{\alpha - Y} \frac{D_{F,e.a.}}{D_{F,e.d.}} \quad \text{electron donor is potentially limiting}$$

$$\frac{S_{LF,e.d.}}{S_{LF,e.a.}} > \frac{1}{\alpha - Y} \frac{D_{F,e.a.}}{D_{F,e.d.}} \quad \text{electron acceptor is potentially limiting}$$

where $S_{LF,e.d.}$ and $S_{LF,a.d}$ are the electron donor and acceptor concentrations, respectively, at the biofilm surface [mg L^{-1}], α is a stoichiometric factor linking the electron acceptor and donor utilization in the catabolic reaction [gO2 gN^{-1}], Y is the biomass yield [gCOD gN^{-1}] and $D_{F,e.d.}$ and $D_{F,e.a.}$ are the diffusion coefficients [m^2 d^{-1}]. Following Chen et al. (2020), we assumed $D_F = 0.8 \cdot D_W$. We used the parameters given in Chen et al. (2020). The results show that for AOB, the electron acceptor, i.e., oxygen, was limiting both in full-scale operation and in the experiments. For NOB, the electron donor, i.e., nitrite, was limiting both in full-scale operation and in the experiments.

Table S1: Parameters for calculations of the limiting compounds and results.

		AOB	**NOB**
α	[mgO_2 mgN^{-1}]	3.4	1.1
Y	[mgCOD mgN^{-1}]	0.22	0.22
$D_{\_W,e.d.}$ (NH_4^+, NO_2^-, respectively)	[m^2 d^{-1}]	$169.1 \cdot 10^{-6}$	$165.2 \cdot 10^{-6}$
$D_{\_W,e.a.}$ (O_2)	[m^2 d^{-1}]	$209.1 \cdot 10^{-6}$	$209.1 \cdot 10^{-6}$
Full-scale operation: $S_{LF,e.d.}$	[mgN L^{-1}]	12 [1)]	0.3 [2)]
Full-scale operation: $S_{LF,e.a.}$	[mgO_2 L^{-1}]	2.5 [3)]	2.5 [3)]
Experiment: $S_{LF,e.d.}$	[mgN L^{-1}]	50 [4)]	0.3 [2)]
Experiment: $S_{LF,e.a.}$	[mg O_2 L^{-1}]	8.3 [5)]	8.3 [5)]
Calculation results:			
$\frac{\mathbf{1}}{\boldsymbol{\alpha - Y}} \frac{\mathbf{D_{F,e.a.}}}{\mathbf{D_{F,e.d.}}}$	[-]	0.4	1.4
Full-scale operation: $\frac{\mathbf{S_{LF,e.d.}}}{\mathbf{S_{LF,e.a.}}}$	[mgN mgO_2^{-1}]	4.8	0.1
Experiment: $\frac{\mathbf{S_{LF,e.d.}}}{\mathbf{S_{LF,e.a.}}}$	[mgN mgO_2^{-1}]	6.0	0.04

[1)] 1/3 of the average influent NH_4^+ concentration (volume exchange ratio at Kloten–Opfikon WWTP = 1/3)

[2)] nitrite effluent concentration at Kloten–Opfikon WWTP and in the experiments was usually below 0.3 mgN L^{-1} under normal operation.

[3)] operating DO concentration at Kloten–Opfikon WWTP

[4)] ammonium spike added to the experiments

[5)] saturation oxygen concentration due to constant aeration

S2 Molecular work

S2.1 16S rRNA gene amplicon sequencing

MLSS was collected from the reactor and separated into flocs (<0.25 mm), small granules (0.25 – 1 mm), medium granules (1 – 2 mm) and large granules (>2 mm) by sieving. Then, water was removed by centrifugation and samples were stored at -80 °C until further processing. Nucleic acid extraction and amplicon sequencing were done at the Genetic Diversity Center (GDC) at ETH Zürich. DNA was extracted using the FastDNA® Spin Kit for Soil (MP Biomedical) following the manufacturer's recommendation except bead-beating was done 4 times at 4 °C at 6 m/s at (with 2-min rest in between) using a bead ruptor (OMNI International). 50 – 150 mg of wet biomass was used for each extraction. A negative control was included for which the same extraction procedure was done but without biomass material (extraction negative control). The PCR primers, purchased from the Integrated DNA Technologies, consist of the Illumina Nextera overhang adapter sequences and the 27F/524R primer set ("Evaluation of 16S rDNA-based community profiling for human microbiome research," 2012) for targeting the V1-V3 16S rRNA gene. To increase the complexity of the sequencing run, four primer sets with varying frame shift(s) plus linkers (Table S2) were mixed in equal amount to yield a final primer set for PCR reaction. Unless specified otherwise, we performed all PCR and qPCR reactions using a SensoQuest thermocycler and Roche Lightcycler 480, respectively. All PCR reactions were conducted using the KAPA HiFi HotStart ReadyMix (Roche). Concentrations of nucleic acids were measured using the Qubit BR or HS dsDNA assays (Invitrogen) on a Qubit fluorometer (Thermo Fisher Scientific) or a Spark 10M Plate Reader (Tecan). All normalization and pooling were done using a Pipetting Robot (BRAND, Germany). All cleanups of PCR products and libraries were done with the AMPure beads (Beckman Coulter) on a KingFisher Apex (ThermoFisher) following the manufacturer's recommendations.

Concentration of the DNA extractions were quantified and normalized to 10 ng/µl. A gradient PCR run was first conducted to determine the optimal annealing temperature, and a qPCR run was conducted to determine the optimal number of cycles for constructing the library. Then, a limited-cycle PCR was done in triplicate 25-µl reactions (12.5 µl KAPA ReadyMix, 0.75 µl each of 10 µM forward/reverse primer, 1 µl of 10 ng/µl DNA template, and 10 µl PCR water) using the following thermal profile: 95°C/3m, 21×(98°C/20s, 62°C /30s, 72°C/30s), 72°C/10min, 4°C/∞. The triplicate PCR reactions were pooled, cleaned up, barcoded with the Nextera XT index adaptors in another PCR run of 25-µl reactions (12.5 µl KAPA ReadyMix, 2.5 µl each of Index 1 and Index 2 primer, 2 µl of the product from limited-cycle PCR, and 5.5 µl PCR water) using the following thermal profile: 95°C/3m, 10×(95°C/30s, 55°C /30s, 72°C/30s), 72°C/5min, 4°C/∞. The PCR products were cleaned up, quantified, and pooled using 39.7 ng of each product. The pooled library was checked using a 4150 TapeStation (Agilent) to verify a single amplicon of 648 bp average size. To avoid under quantification of the

dsDNA due to bubble products, a reconditioning PCR run was performed in four 25-µl replicate reactions (12.5 µl KAPA ReadyMix, 1 µl each of 100 µM Illumina P5 and P7 primer, 1 µl of pooled library, and 9.5 µl PCR water) using the following thermal profile: 95°C/3m, 4×(98°C/20s, 62°C /15s, 72°C/30s), 72°C/1m, 4°C/∞. The products were pooled, and the concentration of the final library was measured again and diluted to 3.82 nM. 15 pM was loaded and sequenced on the MiSeq using the 600PE v3 kit.

Raw sequences analyses were performed by the GDC following their established workflow. Using USEARCH v11.0.667 (Edgar, 2010), forward/reverse reads were merged and primer sequences were stripped using the fastq_mergepairs and search_pcr commands, respectively. Quality filtering was done using PRINSEQ-lite v0.20.4. Zero operational taxonomic units (ZOTUs) were obtained using USEARCH - UNOISE3 (Edgar, 2016b), then further clustered at 97% identity. The reads were then mapped to the ZOTUs with an identity threshold of 97%. Taxonomy of the ZOTUs were assigned against the MiDAS 5.2 (Dueholm et al., 2024) database using the USEARCH - SINTAX classifier (Edgar, 2016a).

Table S2: List of primers used for amplicon sequencing. The heighted sequence indicate the 27F/524R primer set.

Name	Primer Sequence (5' - 3')
27F_nex0	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACAGAGTTTGATCCTGGCTCAG
27F_nex1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNACAGAGTTTGATCCTGGCTCAG
27F_nex2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNACAGAGTTTGATCCTGGCTCAG
27F_nex3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNACAGAGTTTGATCCTGGCTCAG
534R_nex0	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAATTACCGCGGCTGCTGG
534R_nex1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNGAATTACCGCGGCTGCTGG
534R_nex2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNGAATTACCGCGGCTGCTGG
534R_nex3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNGAATTACCGCGGCTGCTGG

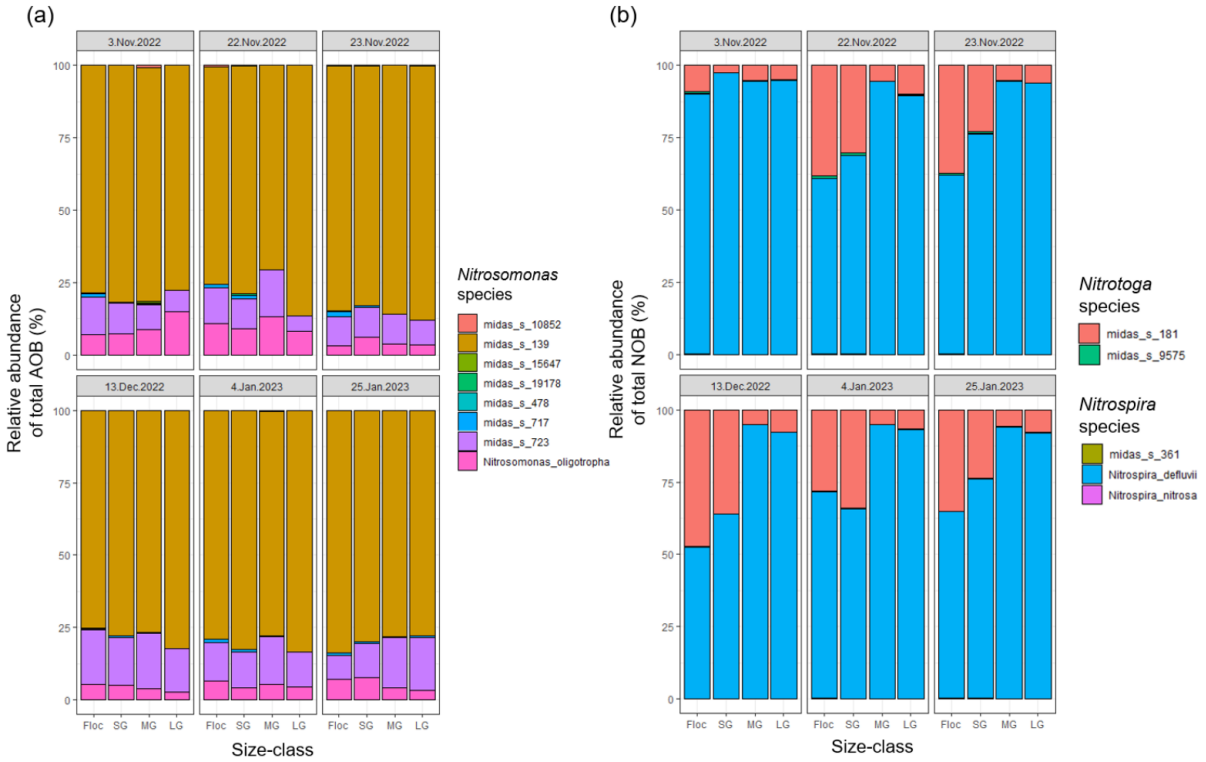

Figure S1: Composition of (a) *Nitrosomonas* species (AOB) and (b) *Nitrotoga* and *Nitrospira* species (NOB) at Kloten–Opfikon WWTP. *Nitrosospira* AOB and *Nitrobacter* NOB were not detected. SG = small granules; MG = medium granules; LG = large granules.

S3 SRT_{min} sensitivity

Table S3: Parameters for SRT_{min} calculation.

		AOB	NOB
Kinetic parameters (SUMO2, Dynamita)			
$u_{max,20°C}$	[d^{-1}]	0.9	0.65
$b_{20°C}$	[d^{-1}]	0.17	0.15
K_N	[mgN L^{-1}]	0.7	0.1
K_{O2}	[mgO_2 L^{-1}]	0.25	0.25
$\theta_{\mu max}$	[-]	1.072	1.06
θ_b	[-]	1.03	1.03
Kinetic parameters (Metcalf et al., 2014)			
$u_{max,20°C}$	[d^{-1}]	0.9	1
$b_{20°C}$	[d^{-1}]	0.17	0.16
K_N	[mgN L^{-1}]	0.5	0.2
K_{O2}	[mgO_2 L^{-1}]	0.5	0.9
$\theta_{\mu max}$	[-]	1.063	1.063
θ_b	[-]	1.026	1.026
Electron acceptor/donor bulk concentrations			
S_N (NH_4+ or NO_2^-)	[mgN L^{-1}]	12*	0.3**
S_{O2}	[mgO_2 L^{-1}]	2.5***	2.5***

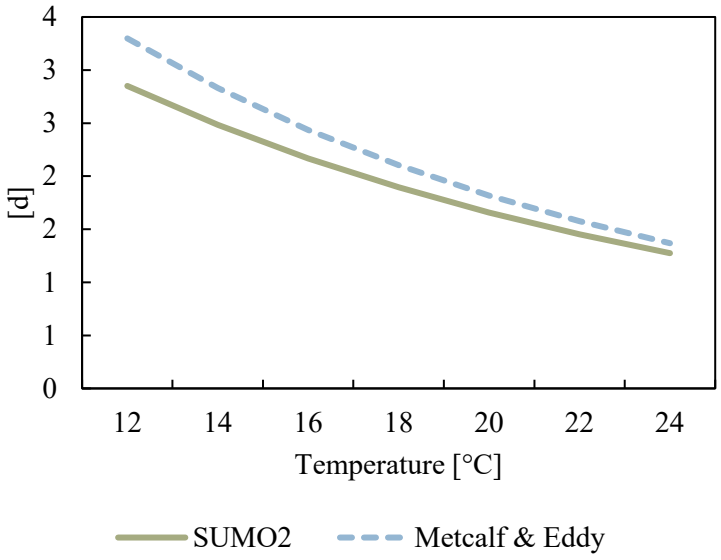

Figure S2: SRT_{min} values of AOB for temperatures between 12 and 24°C for the biokinetic values from SUMO2 (Dynamita) and Metcalf et al. (2014).

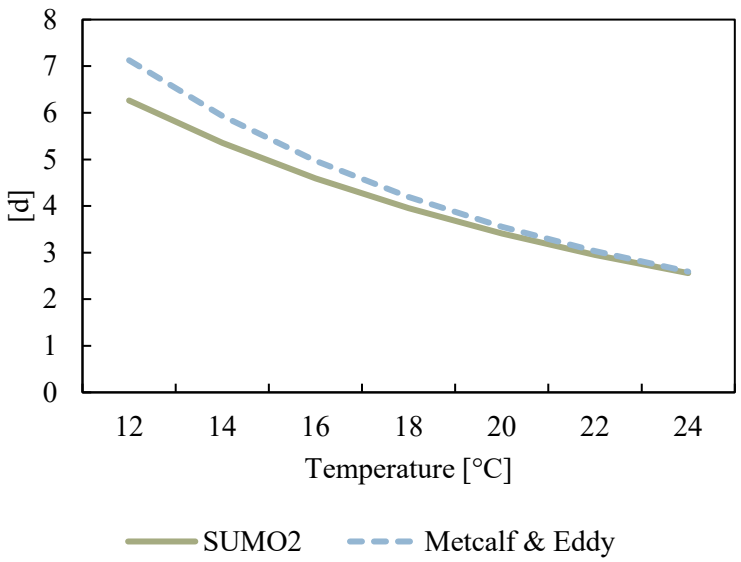

Figure S3: SRT_{min} values of NOB for temperatures between 12 and 24°C for the biokinetic values from SUMO2 (Dynamita) and Metcalf et al. (2014).

S4 Stereomicroscopic image of AGS from Kloten-Opfikon

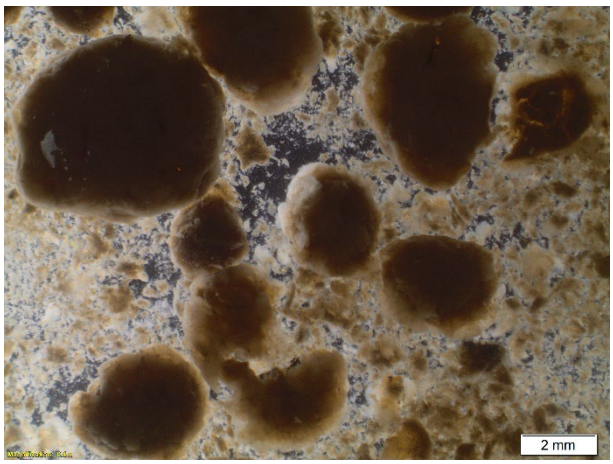

Figure S4: Stereomicroscopic image of the AGS from a sample of Kloten–Opfikon WWTP from August 2023.

Bibliography

Chen, G., Ekama, G. A., van Loosdrecht, M. C. M., & Brdjanovic, D. (2020). *Biological Wastewater Treatment: Principles, Modeling and Design*. IWA Publishing. https://doi.org/10.2166/9781789060362

Dueholm, M. K. D., Andersen, K. S., Korntved, A.-K. C., Rudkjøbing, V., Alves, M., Bajón-Fernández, Y., Batstone, D., Butler, C., Cruz, M. C., Davidsson, Å., Erijman, L., Holliger, C., Koch, K., Kreuzinger, N., Lee, C., Lyberatos, G., Mutnuri, S., O'Flaherty, V., Oleskowicz-Popiel, P., . . . Nielsen, P. H. (2024). MiDAS 5: Global diversity of bacteria and archaea in anaerobic digesters. *Nature Communications*, *15*(1), 5361. https://doi.org/10.1038/s41467-024-49641-y

Dynamita. *SUMO2*. https://dynamita.com/

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460-2461. https://doi.org/10.1093/bioinformatics/btq461

Edgar, R. C. (2016a). SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. *bioRxiv*, 074161. https://doi.org/10.1101/074161

Edgar, R. C. (2016b). UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *bioRxiv*, 081257. https://doi.org/10.1101/081257

Evaluation of 16S rDNA-based community profiling for human microbiome research. (2012). *PLoS One*, *7*(6), e39315. https://doi.org/10.1371/journal.pone.0039315

Metcalf, L., Eddy, H. P., Abu-Orf, M., Bowden, G., Burton, F., Pfrang, W., Stensel, H., Tchobanoglous, G., & Tsuchihashi, R. (2014). AECOM (2014) Wastewater engineering: treatment and resource recovery. In: McGraw Hill Education.