Supplemental Material (SM)

New methods for analysis of spatial distribution and co-aggregation of microbial populations in complex biofilms

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The Slicer algorithm

Morphological closing is applied to the binarized biomass image (Fig. S1A) with a rectangle-shaped structuring element, whose longer edge is parallel to the major orientation axis of the biofilm.

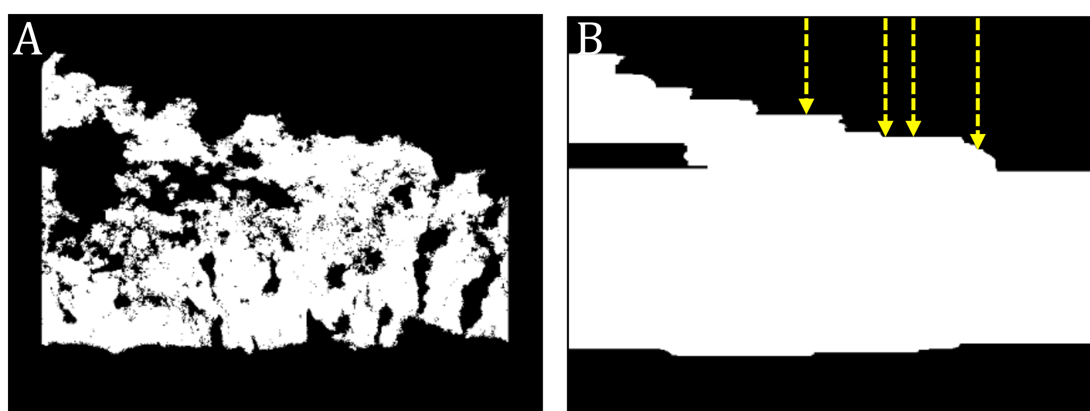


Fig. S1. *Baseline finding step of the automated slicing algorithm.* ***A.*** *A binarized biofilm image (the same as in Fig. 2B).* ***B.*** *The same image as in (A) after morphological closing. Most holes and invaginations of the biofilm have disappeared, and the top and bottom surface lines have been smoothened. The yellow arrows illustrate how this image is scanned to find the baseline for slicing, which in this example is the top surface line of the biofilm.*

For instance, if the surface and base of the biofilm stretch along the *x*-axis of the image, the rectangular structuring element has a long edge parallel to the *x*- and a short edge parallel to the *y*-axis of the image. The closing operation smoothens the surface lines of the biofilm and fills holes and invaginations (Fig. S1 B). The degree of smoothing is determined by the size of the rectangular structuring element: a large structuring element causes pronounced smoothing, whereas a small one preserves

surface invaginations or bulges of the biofilm (compare Fig. 1 A-C). Subsequently, the algorithm defines the "baseline" for slicing, which in the case of a horizontally stretched biofilm (as shown in Fig. 2) can be either the top or the bottom surface line. The baseline is found by scanning the image, pixel by pixel, in a direction that is perpendicular to the major orientation axis of the biofilm. At each position scanning starts at the edge of the image and stops once biomass has been encountered (Fig. S1 B). Starting at the baseline, the algorithm then virtually sections the original binary image (i.e., the image before the closing operation) in slices of a user-defined thickness. The edges of these slices have the same contour as the baseline to ensure that the thickness of a slice is constant at all positions. The resulting binary image contains the biomass and the edges of all slices (Fig. 2C). This image is called the "slicer template". Subsequently, the software can cut the original FISH images (showing the signals of the EUB probe mix or of specific probes; Fig. 2A and D) into slices as defined by the slicer template (Fig. 2E and F). Thus, the template is defined only once and then applied to slice an arbitrary number of FISH images that show the same biofilm region but different probe signals. If the FISH images have already been segmented, the segmentation data (=object definitions) are transferred automatically to the sliced sub-images (Fig. 2D and E). Slicer templates can be created in one run for many biofilm images that show different biofilm regions (FOVs). Such batches of templates can then be used to slice the corresponding batches of FISH images showing different probe signals.

Fluorescence in situ hybridization

Probe sequences and hybridization conditions are listed in Table S1.

Table S1. Oligonucleotide probes used for fluorescence in situ hybridization (FISH).

Probe	Sequence (5'-3')	Position[a]	Target	FA (%)	Ref.
Eub338[b]	GCTGCCTCCCGTAGGAGT	338-355	Most *Bacteria*	0-50	(1) [b]
Bet42a[d]	GCC TTC CCA CTT CGT TT	1027-1043 (23S rRNA)	Betaproteobacteria	35	(2)
Cluster6a192[c,e]	CTTTCGATCCCCTACTTTCC	192-211	*N. oligotropha* lineage	35	(3)
Noli191[d,e,f]	CGATCCCCCACTTTCCTC	191-208	*N. oligotropha* lineage	35[f]	(4) [f]
Nmo218	CGG CCG CTC CAA AAG CAT	218 - 235	*N. oligotropha* lineage	35	(4)
Neu[d,e,f]	CCCCTCTGCTGCACTCTA	653 - 670	Most halophilic and halotolerant *Nitrosomonas spp.*	35[f]	(5) [f]
Nso1225[e]	CGCCATTGTATTACGTGTGA	1224 - 1243	Most beta-proteobacterial AOB	35	(6)
Nse1472[g]	ACCCCAGTCATGACCCCC	1472 - 1489	*Nitrosomonas europea*, *N. halophila*, *N. eutropha,* Kraftisried-Isolat Nm103	50	(7)
Ntspa662[d]	GGAATTCCGCGCTCCTCT	662 - 679	genus *Nitrospira*	35	(8, 9)
Ntspa 1151	TTC TCC TGG GCA GTC TCT CC	1151 - 1170	Sublineage II of the genus *Nitrospira*	35-40	(10)

[a] *E. coli 16S rRNA position (11)*

[b] *Used in mixture with Eub338-II and Eub338-III (the Eub338 probe mix) (8)*

[c] *Used together with an unlabeled competitor oligonucleotide as indicated in the reference. Competitor not included in AOB probe-mix.*

[d] *Used together with an unlabeled competitor oligonucleotide as indicated in the reference.*

[e] *Used in the AOB probe-mix only*

[f] *FA concentrations in hybridization buffer adjusted from 30% FA, (Noli191) and 40% FA, (Neu) for use in the AOB probe-mix.*

[g] *DOPE-FISH labeled probe as described in (12).*

DNA extraction, construction of clone libraries and sequencing

DNA was extracted, by using the FastDNA spin kit for soil (MP Biomedicals, France), from pooled, triplicate biofilm samples from each of the pilot plant reactors MBBR T1 and NTF2. Extraction and purification of DNA were performed as recommended by the manufacturer.

16S rRNA genes were amplified from MBBR T1 and NTF2 samples by PCR based on the universal bacterial primers 27F and 1492R (13) and on the AOB-specific primer pairs βAMOf - βAMOr (14) and NitA (modified in this study) - NitB (15). The modified primer NitA (5′-CTKAAGTGGGGRATAACGCATCG-3′) returned a higher number of hits to target bacteria within the *Nitrosomonadaceae* than the original NitA (170 vs. 85), while allowing for a few more non-target hits (20 vs. 2) when probed against the Ribosomal Database Project 16S rRNA gene database v10 (data not shown). PCR was carried out in a total reaction volume of 50 µl with 1.25 U HotStarTaq plus DNA polymerase, 5 µl 10 × PCR buffer (Qiagen, Germany), 200 µM of each dNTP, 0.4 µM of each primer, and 2 µl template DNA extract. The PCR protocol for all primers consisted of a hot start (95°C, 5 min), 30 cycles of denaturation (94°C, 45 s), annealing (52°C, 45 s), and elongation (72°C, 1 min 45 s), followed by a final elongation (72°C, 7 min) using a Biometra T3000 thermocycler (Biometra, Germany). All PCR reactions were performed in triplicates. The triplicate PCR products were pooled and purified using QIAquick columns (Qiagen), and the amplicon size was assessed by agarose gel electrophoresis. Clone libraries were made using the TOPO TA cloning kit for sequencing (Invitrogen) as recommended by the manufacturer. In total 96 clones from each library were randomly selected for colony PCR using the M13F-20 and M13R vector primers. The universal (27F - 1492R)

cloned 16S rRNA genes were first partially sequenced using the primer 341F (16). A subset of these genes was selected for further (nearly full-length) sequencing with the primers M13F-20 and M13R, together with the AOB specific (βAMOf - βAMOr and NitA (mod) – NitB) cloned 16S rRNA genes. Sequencing was performed by Macrogen Inc. (Seoul, South Korea).

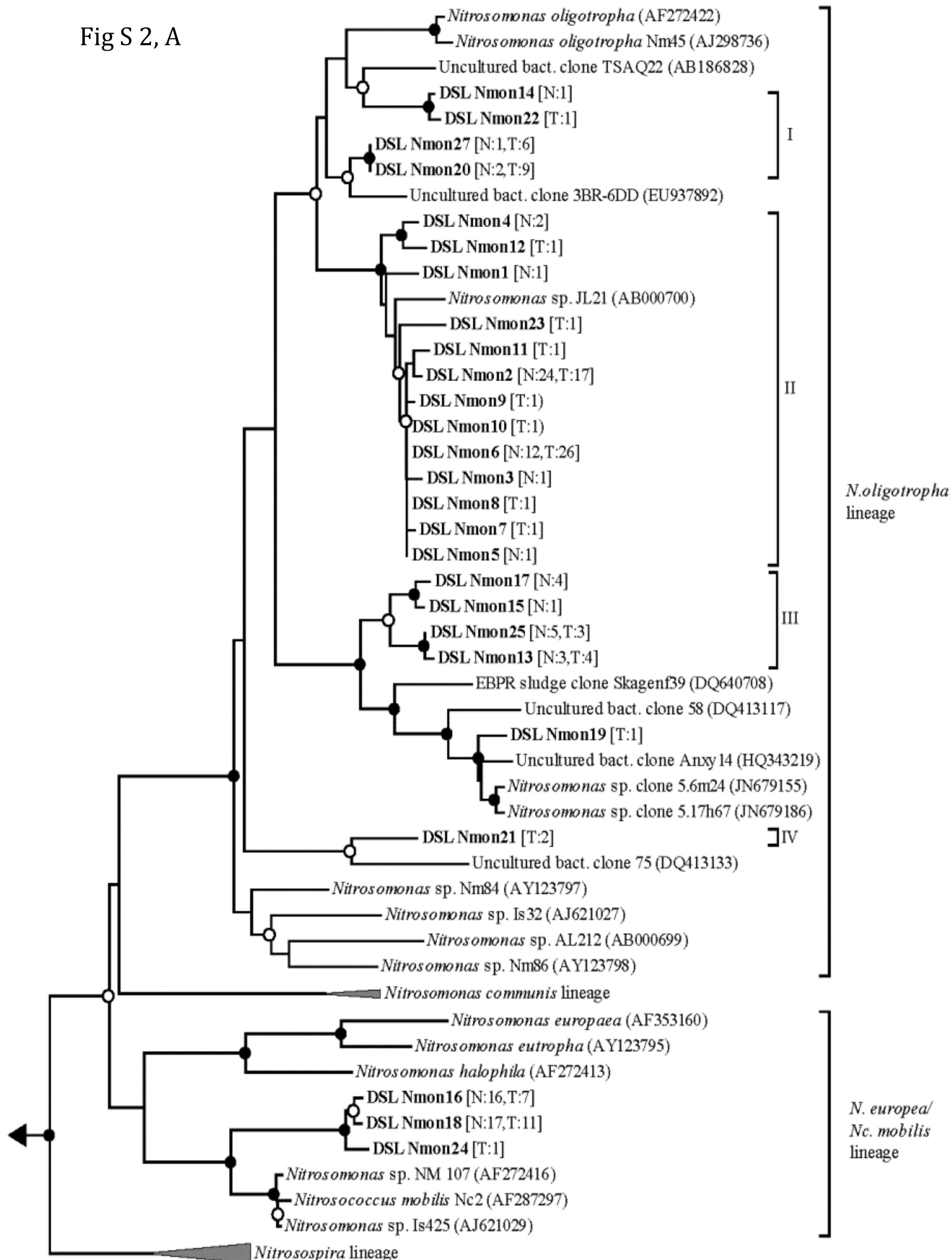
Phylogenetic analyses

Sequences were assembled and trimmed using DNA Baser v2.9 (Heracle BioSoft) prior to alignment using the NAST algorithm in Greengenes (17). Putative chimeric sequences, as identified by Bellerophon (18) were excluded from subsequent analyses. Sequences were grouped according to a 99% identity threshold. Phylogenetic trees were constructed based on the neighbor-joining method and Kimura two-parameter model using the MEGA5 software (19). The strength of support for tree branches was assessed by bootstrapping with 1,000 iterations.

Nitrifying community composition

In total, 186 16S rRNA gene sequences of AOB and 20 sequences of NOB were retrieved from a pilot-scale trickling filter (NTF2) and a moving bed biofilm reactor (MBBR T1) and subjected to phylogenetic analysis (Fig. S2). The majority (134) of th AOB sequences fell into the *Nitrosomonas oligotropha* lineage (cluster 6a) (20). Among these sequences, four phylogenetic subclusters could be distinguished that contained at least two clones each and also differed in their sequences at the target sites of the *N. oligotropha*-specific FISH probes Nmo218 and Cluster6a192 (Fig. S2, Table S2). One of these groups, here referred to as *N. oligotropha* subcluster I, had fully matching target sites for both Nmo218 and Cluster6a192. The second group,

subcluster II, matched only Cluster6a192 but had two sequence mismatches to probe Nmo218.



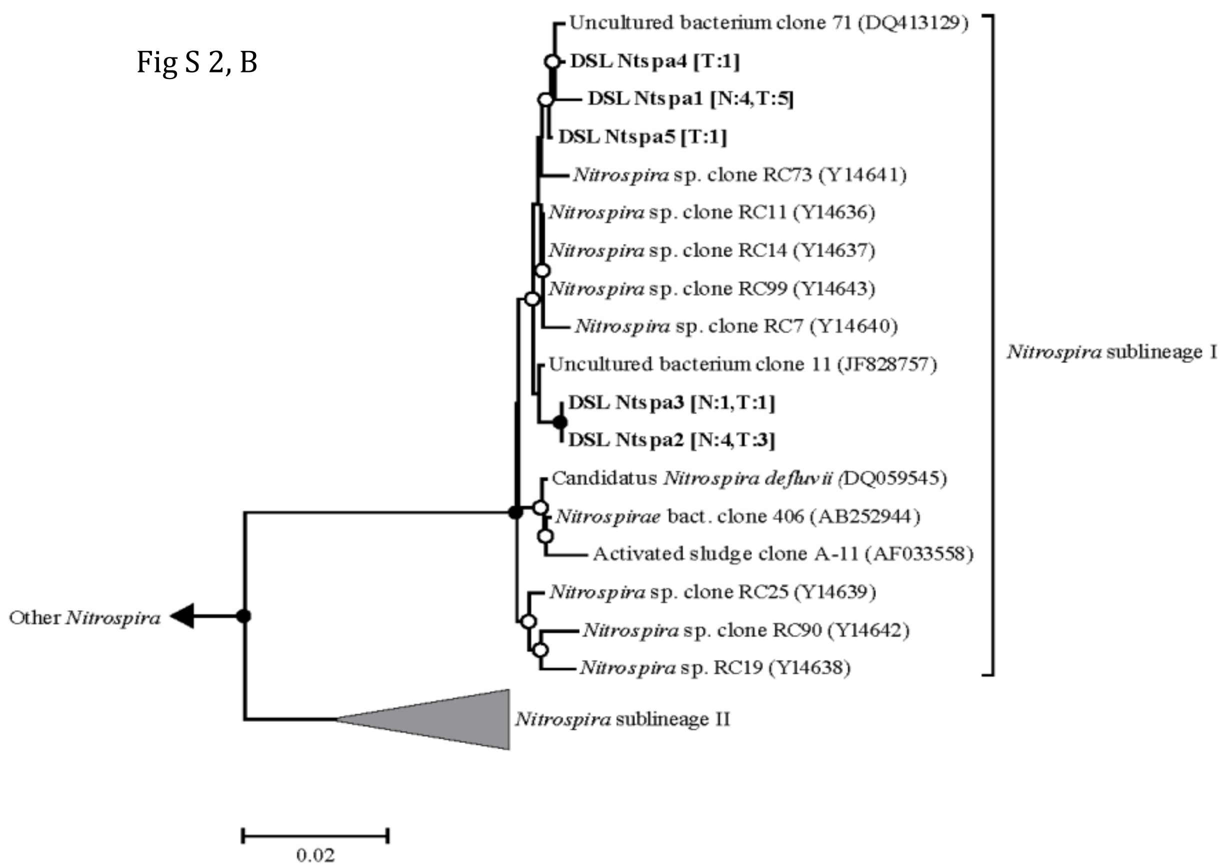


Fig. S2. *Phylogenetic relationships of the 16S rRNA gene sequences for ammonia-oxidizing bacteria (A) and nitrite oxidizing bacteria (B). The number of clones with > 99% sequence similarity is shown in brackets, where N and T represent clones from NTF2 and MBBR T1, respectively. The trees were constructed using the neighbor joining method with 1000 replicate bootstraps. Bootstrap values > 50% and >90% are indicated by empty and filled circles, respectively. The scale bar represents number of nucleotide substitutions per site. Subclusters of ammonia-oxidizing bacteria within the N. oligotropha lineage are shown by brackets (I-IV).*

The sequences forming subcluster III had one or two mismatches to the probes Cluster6a192 and Nmo218, suggesting that these AOB are not detected by either of these probes (Table S2). Subcluster IV was detected only in reactor MBBR T1 and fully matched the sequence of probe Nmo218, but had one mismatch to Cluster6a192.

Subcluster	Sequence	Cluster6a 192	Nmo218	NEU
	Target site	GGAAAGUAGGGGAUCGAAAG	AUGCUUUUGGAGCGGCCG	UAGAGUGCAGCAGAGGGG
I	DSL Nmon20	--------------------	------------------	-------U---------A
I	DSL Nmon22	--------------------	------------------	-------UG---------
I	DSL Nmon27	--------------------	------------------	-------U---------A
II	DSL Nmon2	--------------------	-------AA---------	-------U---------A
II	DSL Nmon6	--------------------	-------AA---------	-------U---------A
II	DSL Nmon12	--------------------	-------AA---------	-------U---------A
III	DSL Nmon13	-A--------A---------	GC----------------	-------U-A-------A
III	DSL Nmon17	-A------------------	-C----------------	-------U-A-------A
III	DSL Nmon25	-A------------------	GC----------------	-------U-A-------A
	DSL Nmon19	-A------------------	GC----------------	-------U-A-------A
IV	DSL Nmon21	-A------------------	------------------	-------UG---------
	DSL Nmon16	-U-----G--------C---	GC----GA----------	-------U----------
	DSL Nmon18	-U-----G--------C---	GC----GA----------	-------U----------
	DSL Nmon24	-U-----G--------C---	GC----GA----------	-------U----------

Table S2. *Target site sequences (16S rRNA) of selected phylotypes to the FISH probes used to distinguish between ammonia-oxidizing bacteria in this study. The different subclusters within the Nitrosomonas oligotropha lineage, as defined in this study, are also specified.*

To check whether the phylogenetic subclusters I, II, and IV indeed were present *in situ* in the biofilms, FISH experiments were performed with biomass from reactors NTF2 and MBBR T1 and the probes Cluster6a192 and Nmo218, which were applied simultaneously but labeled with different fluorochromes. Evaluation by confocal laser scanning microscopy confirmed that, based on the probe binding patterns, subclusters I and II occurred in the samples whereas subcluster IV was not detected *in situ*. As no

probe binding pattern would be specific for subcluster III, the presence of this lineage was not verified by FISH. Furthermore, 52 sequences were related to the *Nitrosomonas europaea/Nitrosococcus mobilis* lineage (cluster 7) (20). The presence of these AOB in the biofilms was confirmed by FISH with probe Nse1472 targeting *N. europaea* and closely related organisms (Fig. 1 and Table 1).

A total of 20 NOB sequences were obtained and were closely related to "*Candidatus* Nitrospira defluvii" (21) belonging to the sublineage I of the genus *Nitrospira* (9) (Fig S2, B). All sequences fully matched the target site of probe Ntspa662 specific for the genus *Nitrospira*. Although not detected by cloning and sequencing, a small number of *Nitrospira* colonies in reactor MBBR T1 hybridized to probe Ntspa1151, specific for sublineage II of the genus *Nitrospira* (10). Other known NOB were not detected, suggesting that this community in the analyzed reactors consisted solely of *Nitrospira*. These results are consistent with previous studies of the same pilot plant (22, 23).

FISH and 16S rRNA gene sequence data showed, in accordance with earlier investigations (22-24) that *Nitrospira* within sublineage I dominated the NOB guild in the biofilms analyzed (Fig. 1B, Fig. 2). The AOB communities, on the other hand, were rather diverse and at least five 16S rRNA gene sequence subclusters were found in the NTF and MBBR systems. The diversity within the nitrifier guild, especially the AOB, has been suggested to be important for resilience of the nitrification process in wastewater treatment (9, 20, 25-27). This may, however, not necessarily mean diversity at the species level, as intra-lineage diversity can be considerable (22, 28). Among the AOB in this study, one subcluster was affiliated to the *N. europaea*/*Nc. mobilis* lineage and four to the *N. oligotropha* lineage (Fig. S1A). This observed *N. oligotropha* intra-lineage diversity suggests that the AOB in the pilot-plant system

were more diverse than previously demonstrated by using FISH and DGGE (22). Even more pronounced intra-lineage diversity within *N. oligotropha* was recently demonstrated in a wastewater membrane bioreactor system, where as many as 13 distinct OTUs within *N. oligotropha*, were discovered (28). Therefore, further insights into the ecophysiology of AOB and NOB may show functional redundancy also in nitrifying systems with a low diversity at the level of deep-branching lineages if niche partitioning among very closely related nitrifiers is significant.

Vertical distribution pattern of Nitrospira in the Full-Scale NT

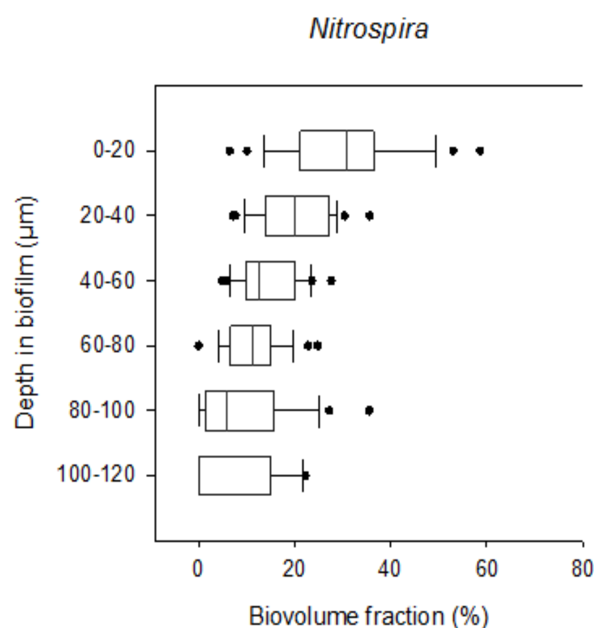


Fig. S3. *Boxplot showing the vertical distribution pattern of Nitrospira in the Full-Scale NTF as determined by the automated Slicer when applied on the* ***original*** *CLSM micrograph field of views (FOVs) (n=30) from (24). Relative abundance is expressed as the percentage of the total bacterial community as determined by the EUB338 probe mix, at different depths in the biofilm.*

References for Supplemental

1. **Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919-1925.
2. **Manz W, Amann R, Ludwig W, Wagner M.** 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst. Appl. Microbiol. **15:**593-600.
3. **Adamczyk J, Hesselsoe M, Iversen N, Horn M, Lehner A, Nielsen PH, Schloter M, Roslev P, Wagner M.** 2003. The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. Appl. Environ. Microbiol. **69:**6875-6887.
4. **Gieseke A, Purkhold U, Wagner M, Amann R, Schramm A.** 2001. Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Appl. Environ. Microbiol. **67:**1351-1362.
5. **Wagner M, Rath G, Amann R, Koops H-P, Schleifer K-H.** 1995. In situ Identification of Ammonia-oxidizing Bacteria. Syst. Appl. Microbiol. **18:**251-264.
6. **Mobarry BK, Wagner M, Urbain V, Rittmann BE, Stahl DA.** 1997. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Erratum*. Appl. Environ. Microbiol. **63:**815.
7. **Juretschko S, Timmermann G, Schmid M, Schleifer K-H, Pommerening-Roser A, Koops HP, Wagner M.** 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations. Appl. Environ. Microbiol. **64:**3042-3051.
8. **Daims H, Brühl A, Amann R, Schleifer K-H, Wagner M.** 1999. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. **22:**434-444.
9. **Daims H, Nielsen JL, Nielsen PH, Schleifer K-H, Wagner M.** 2001. In situ characterization of Nitrospira-like nitrite-oxidizing bacteria active in wastewater treatment plants. Appl. Environ. Microbiol. **67:**5273.
10. **Maixner F, Noguera DR, Anneser B, Stoecker K, Wegl G, Wagner M, Daims H.** 2006. Nitrite concentration influences the population structure of Nitrospira-like bacteria. Environ. Microbiol. **8:**1487-1495.
11. **Brosius J, Palmer ML, Kennedy PJ, Noller HF.** 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. **75:**4801-4805.
12. **Stoecker K, Dorninger C, Daims H, Wagner M.** 2010. Double labeling of oligonucleotide probes for fluorescence in situ hybridization (DOPE-FISH) improves signal intensity and increases rRNA accessibility. Appl. Environ. Microbiol. **76:**922-926.
13. **Lane DJ.** 1991. 16S/23S rRNA sequencing., p. 115-175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics John Wiley and Sons Ltd,, Chichester.
14. **McCaig aE, Embley TM, Prosser JI.** 1994. Molecular analysis of enrichment cultures of marine ammonia oxidisers. FEMS Microbiology Letters **120:**363-367.
15. **Voytek MA, Ward BB.** 1995. Detection of Ammonium-Oxidizing Bacteria of the Beta-Subclass of the Class Proteobacteria in Aquatic Samples with the PCR. . Appl. Environ. Microbiol. **61:**1444-1450.

16. **Muyzer G, de Waal EC, Uitterlinden aG.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695-700.
17. **DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL.** 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. **72:**5069-5072.
18. **Huber T, Faulkner G, Hugenholtz P.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. . Bioinformatics **20:**2317–2319.
19. **Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S.** 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **28:**2731-2739.
20. **Purkhold U, Pommerening-Roser A, Juretschko S, Schmid MC, Koops HP, Wagner M.** 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. **66:**5368-5382.
21. **Spieck E, Hartwig C, McCormack I, Maixner F, Wagner M, Lipski A, Daims H.** 2006. Selective enrichment and molecular characterization of a previously uncultured Nitrospira-like bacterium from activated sludge. Environ. Microbiol. **8:**405-415.
22. **Lydmark P, Almstrand R, Samuelsson K, Mattsson A, Sorensson F, Lindgren P-E, Hermansson M.** 2007. Effects of environmental conditions on the nitrifying population dynamics in a pilot wastewater treatment plant. Environ. Microbiol. **9:**2220-2233.
23. **Almstrand R, Lydmark P, Sorensson F, Hermansson M.** 2011. Nitrification potential and population dynamics of nitrifying bacterial biofilms in response to controlled shifts of ammonium concentrations in wastewater trickling filters. Bioresour. Technol. **102:**7685-7691.
24. **Lydmark P, Lind M, Sorensson F, Hermansson M.** 2006. Vertical distribution of nitrifying populations in bacterial biofilms from a full-scale nitrifying trickling filter. Environ. Microbiol. **8:**2036-2049.
25. **Siripong S, Rittmann BE.** 2007. Diversity study of nitrifying bacteria in full-scale municipal wastewater treatment plants. Water Res. **41:**1110-1120.
26. **Rowan AK, Snape JR, Fearnside D, Barer MR, Curtis TP, Head IM.** 2003. Composition and diversity of ammonia-oxidizing bacterial communities in wastewater treatment reactors of different design treating identical wastewater. FEMS Microbiol. Ecol. **43:**195-206.
27. **Wittebolle L, Vervaeren H, Verstraete W, Boon N.** 2008. Quantifying community dynamics of nitrifiers in functionally stable reactors. Appl. Environ. Microbiol. **74:**286-293.
28. **Wan C-Y, De Wever H, Diels L, Thoeye C, Liang J-B, Huang L-N.** 2011. Biodiversity and population dynamics of microorganisms in a full-scale membrane bioreactor for municipal wastewater treatment. Water Res. **45:**1129-1138.