Supporting Information for:

Rapid and Sensitive Quantification of Anammox Bacteria by Flow Cytometric Analysis Based on Catalyzed Reporter Deposition Fluorescence In Situ Hybridization

Yijing Zhu, Yayi Wang*, Yuan Yan, Hao Xue

State Key Laboratory of Pollution Control and Resources Reuse, Shanghai Institute of Pollution Control and Ecological Security, College of Environmental Science and Engineering, Tongji University, Siping Road, Shanghai 200092, P. R. China

*Corresponding author. Tel: +21 65984275; Fax: +21 65984275; E-mail: wyywater@126.com; yayi.wang@tongji.edu.cn

Supporting Information includes 13 pages, 9 figures and 3 tables.

Supplementary materials and methods

In the CARD-FISH of this study, one targeted site on 16S rRNA gene was labeled by one molecule of biotin through hybridization. Then, HRP molecules specifically binds on 16S rRNA genes (30S subunit of ribosome) of anammox bacteria, through the binding of biotin and streptavidin molecules (conjugated with HRP). After that, fluorophores (Alexa Fluor 647 used in this study) were deposited on the targeted site by the reaction of tyramide and HRP molecules.

Flow-CARD-FISH embedded in agarose.

To evaluate the flow cytometry-compatible protocol of CARD-FISH by two forms of in-solution and embedded in agarose, fixed and homogenized samples (1 mL) were pelleted (8000 × *g*, 10 min) and then homogenously mixed and embedded in 1 mL 5% (w/v) melted low-gelling-point agarose (Thermo Scientific). Aliquots of 50 μL were transferred into each well of a 96-well microtiter plate and cooled until forming a gel before subsequent treatment. The subsequent permeabilization, blocking, hybridization and TSA treatment were performed identically as to the aforementioned method ("Flow-CARD-FISH (in-solution) Protocol Optimization" in the main test), except for the rinsing during processing. Specifically, samples embedded in agarose were rinsed by incubating in rising solution for 5–10 min, after which the rinsing solution was carefully wiped with blotting paper. This process was repeated three times. The CARD-FISH hybridized samples embedded in agarose were melted by heating to 65°C, then diluted with 1×PBS for subsequent FCM analysis (Fig. S4).

Comparison of Flow-CARD-FISH with other quantitative methods.

To further evaluate the protocol, the Flow-CARD-FISH method was applied to different anammox sludge samples (Sludge-J and Sludge-K). Each sample was prepared in triplicate and analyzed in duplicate. The quantification results obtained with the proposed Flow-CARD-FISH were compared with the four other quantification methods described below.

Flow cytometric monolabeled FISH (Flow-monolabeled FISH).

A modification of the standard FISH protocol for flow-cytometric measurements was adopted (Fig. S6), and the proposed in-solution FISH protocol was employed without solid support (glass slides, polycarbonate filters) or resuspension of bacteria removed from a solid support. The collected samples were first fixed with a 4% (w/v) solution of paraformaldehyde (Sigma-Aldrich) at 4°C for 12 h, then washed three times with 1 × PBS.

To evaluate the effects of cell wall permeabilization on the intensity of the fluorescence signal of Flow-FISH, one group of samples was treated with lysozyme at different concentrations (0.2 and 0.5 mg·mL^{-1} in 0.1 M Tris-HCl [pH 7.5], Sigma-Aldrich) at 25°C for 1

h, while the other group of samples was not subjected to permeabilization treatment. The negative control probe NON338 and specific probes AMX368, KST1275 and JEC152 labeled at the 3' end with Alexa Fluor 647 fluorophore was used for Flow-FISH. The pellets (8000 × *g*, 10 min) of 50 µL aliquots of samples were resuspended in 50 µL hybridization buffer (900 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.1% SDS, 30% formamide, ultrapure water). Next, 20 µL NON338 and specific probe stock solution (concentrations of 5 µM for low concentration and 20 µM for high concentration) were added to negative control samples and experimental samples, respectively. In-solution hybridization was subsequently performed at 46°C for 2 h on an orbital shaker. Following hybridization, 1 mL prewarmed (48°C) washing buffer (3 mM NaCl, 5 mM EDTA [pH 8.0], 20 mM Tris-HCl [pH 8.0], 0.01% [w/v] SDS) was added to each tube prior to centrifugation (8000 × *g*, 10 min). The pellets were rinsed again with 1 mL of washing buffer to remove unbound probes, then incubated at 48°C for 15 min. Samples were rinsed for three times with precooled (4°C) ultrapure water after centrifugation (8000 × *g*, 10 min).

Monolabeled FISH for epifluorescence microscopy.

The method utilized for epifluorescence analysis was adapted from the standard FISH protocol.[1–3] Briefly, sludge samples were dispersed using an ultrasonic probe and fixed with a solution of 4% (w/v) paraformaldehyde (Sigma-Aldrich) at a ratio of 1:3 v/v at 4°C for 12 h. After rinsing three times with 1 × PBS, samples were diluted with 1 × PBS for immobilization of fixed cells on microscope slides. Next, 10 µL aliquots of bacteria suspensions were loaded onto glass slides (Beyotime, China) and dried for 10 min at 46°C. The bacteria were then dehydrated by successive passage through 50, 80 and 90% ethanol (3 minutes each) and air dried. To each slide, 10 µL of freshly prepared hybridization buffer (900 mM NaCl, 20 mM Tris-HCl [pH 8.0], 0.1% SDS, 30% formamide) and 1 µL probe working solution containing 5 ng of each of the respective dye-labeled probes was added. 3'-Alexa Fluor 647 labeled oligonucleotide probes of AMX368, KST1275 and JEC152 were used. The slides were then incubated at 46°C for 2 h in 50 mL Falcon tubes containing a tissue paper moisturized with 2 mL of hybridization buffer. Following hybridization, the slides were washed rapidly with prewarmed (48°C) washing buffer (3 mM NaCl, 5 mM EDTA [pH 8.0], 20 mM Tris-HCl [pH 8.0], 0.01% [w/v] SDS) before being immersed in the same buffer and incubated at 48°C for 15 min. Slides were rinsed with cold ultrapure water, air dried rapidly and stored at -20°C after counterstaining with DAPI (Thermo Scientific). For epifluorescence microscopy, slides were analyzed with a Nikon A1R confocal microscope (Nikon Instruments Inc., Japan) and at least 20 microscopic fields were counted. Photomicrographs (Fig. 4) were analyzed using the ImageJ imaging software[4] (available at https://imagej.nih.gov/ij/).

16S rRNA sequencing analysis.

Microbial DNA was extracted from AS samples and purified using the E.Z.N.A.® DNA Kit (Omega Bio-tech, Norcross, GA, USA) according to the manufacturer's protocols. PCR amplifications were conducted in reaction mixtures with a total volume of 20 μL using a DNA thermocycler (ABI GeneAmp® 9700, USA). The 16S rRNA genes were amplified from the DNA extracts using the 338F (5'–ACTCCTACGGGAGGCAGCAG–3') and 806R (5'–GGACTACATCGACGGGTATTCTAAT–3') primers.[5] Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions, then quantified using QuantiFluor™-ST (Promega, USA). Purified amplicons were pooled in equimolar amounts and high-throughput sequenced using the Illumina MiSeq platform (Majorbio, Shanghai) according to the standard protocols. The resulting Illumina fastq files were de-multiplexed and quality-filtered using QIIME™ v1.9.1.[6] Operational taxonomic units (OTUs) with a 97% similarity cutoff were clustered using USEARCH (v9.2.64, http://drive5.com/uparse/),[7] and chimeric sequences were identified and removed using UCHIME.[8] Representative sequences of each OTU were then assigned to a taxonomic rank at a 70% threshold on the QIIME platform (http://qiime.org/scripts/assign_taxonomy.html) using the Ribosomal Database Project (RDP) classifier and were allocated to different taxa levels (Fig. S8).

Quantitative PCR (qPCR).

DNA was extracted from sludge samples using the FastDNA® SPIN Kit for Soil (MP-Biomedicals, Santa Ana, USA) according to the manufacturer's protocols. The targeted genes (Table S1) were selected for quantitative detection using the SYBR® Green method.[9] Primer sequences as well as the applied qPCR conditions are summarized in Table S1. qPCRs were conducted in 96-well plates with a final volume of 25 mL consisting of 12.5 mL 2 × Power SYBR® Green PCR Master Mix (Life Technologies, USA), 0.5 mL of each primer (10 mM), 2 mL of template DNA (2 ng·mL^{-1}) and 9.5 mL of ddH_2O. All of the qPCR amplifications and quantification were conducted on an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, USA). The PCR protocol was as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Following each run, a melting curve was generated and analyzed to avoid nonspecific amplification. Each reaction was run in triplicate for each permutation of the sample and primer set, as well as the negative controls, in which ultrapure water was added instead of DNA template. Triplicate anammox sludge samples were collected for qPCR, which could discount the influence of biological variability. Each sample was detected in triplicate to ensure low technical variation. Data analysis provided percentages of anammox 16S rRNA relative to the total bacterial 16S rRNA (Table S2).

Supplementary Tables

Table S1 Primers and thermal cycling conditions for qPCR

Tested genes		Sequence (5'–3')	Amplicon size (bp)	Annealing temperature (°C)	References
Bacterial 16S rRNA	Eub338	ACTCCTACGGGAGGCAGCAG	181	55	
	Eub518	ATTACCGCGGCTGCTGG			
anammox 16S rRNA (AMX368F-AMX820R)	AMX368F	TTCGCAATGCCCGAAAGG	478	58	[10]
	AMX820R	AAAACCCCTCTACTTAGTGCCC			[11]

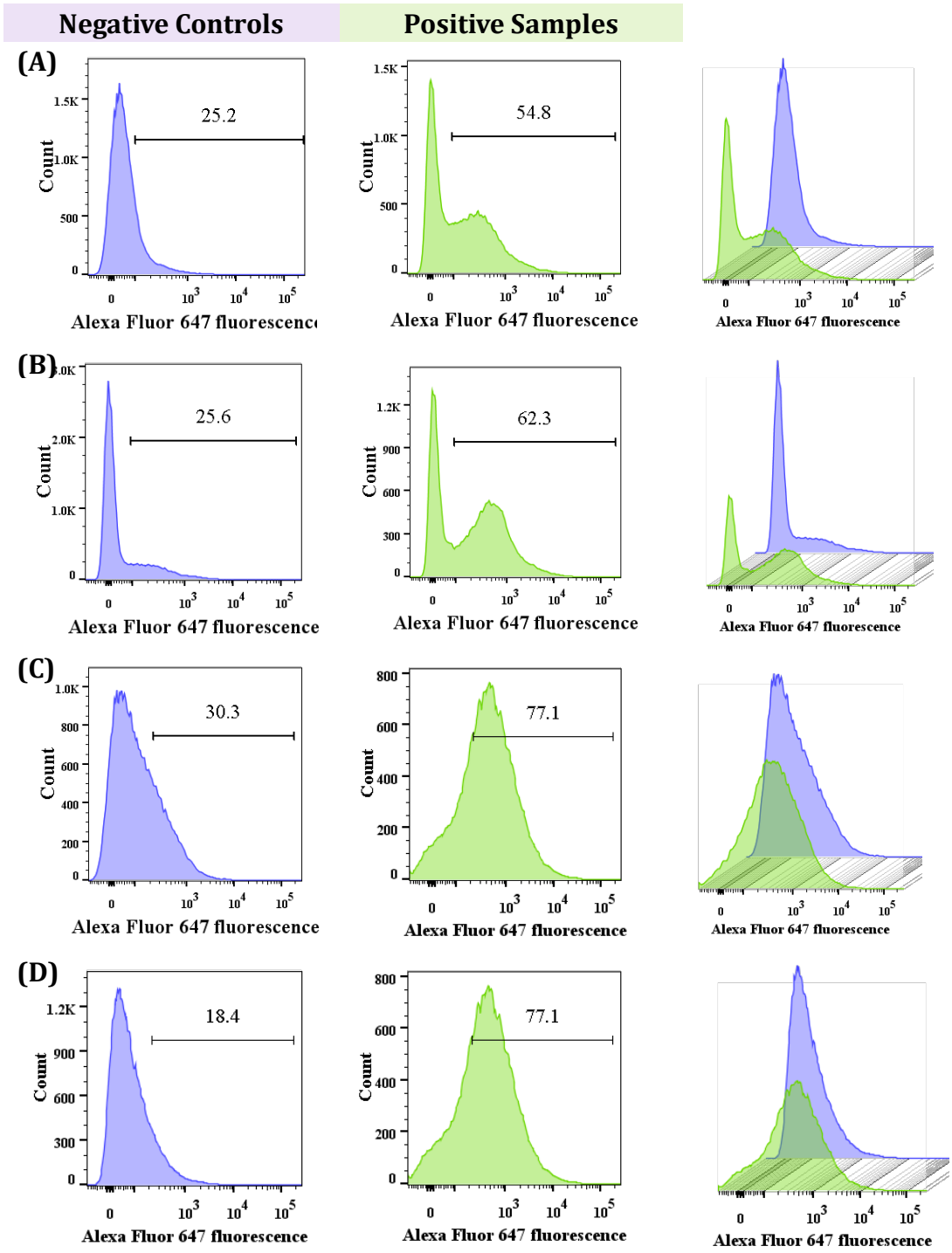
Table S2 Results of qPCR of anammox sludge samples

Sludge Samples	Bacterial 16S gene (copies/g VSS) (mean ± s.d.)	Anammox bacterial 16S rRNA (copies/g VSS) (mean ± s.d.)	Relative proportion of anammox bacteria (mean ± s.d.)
Sludge-J	$6.69 \pm 0.21 \times 10^8$	$2.34 \pm 0.03 \times 10^8$	34.95 ± 2.92 %
Sludge-K	$1.07 \pm 0.07 \times 10^9$	$2.40 \pm 0.04 \times 10^8$	25.13 ± 3.82%

Table S3 Absolute abundance of anammox bacteria in sludge samples analyzed by Flow-CARD-FISH

Sludge Samples	Absolute abundance of anammox bacteria (cells/mL)	
Sludge-J	Total anammox bacteria	$(2.31 \pm 0.03) \times 10^7$
	Candidatus Jettenia	$(2.24 \pm 0.02) \times 10^7$
	Candidatus Kuenenia	$(1.26 \pm 0.11) \times 10^5$
Sludge-K	Total anammox bacteria	$(1.20 \pm 0.06) \times 10^7$
	Candidatus Jettenia	$(9.05 \pm 0.12) \times 10^4$
	Candidatus Kuenenia	$(1.18 \pm 0.05) \times 10^7$

Supplementary Figures



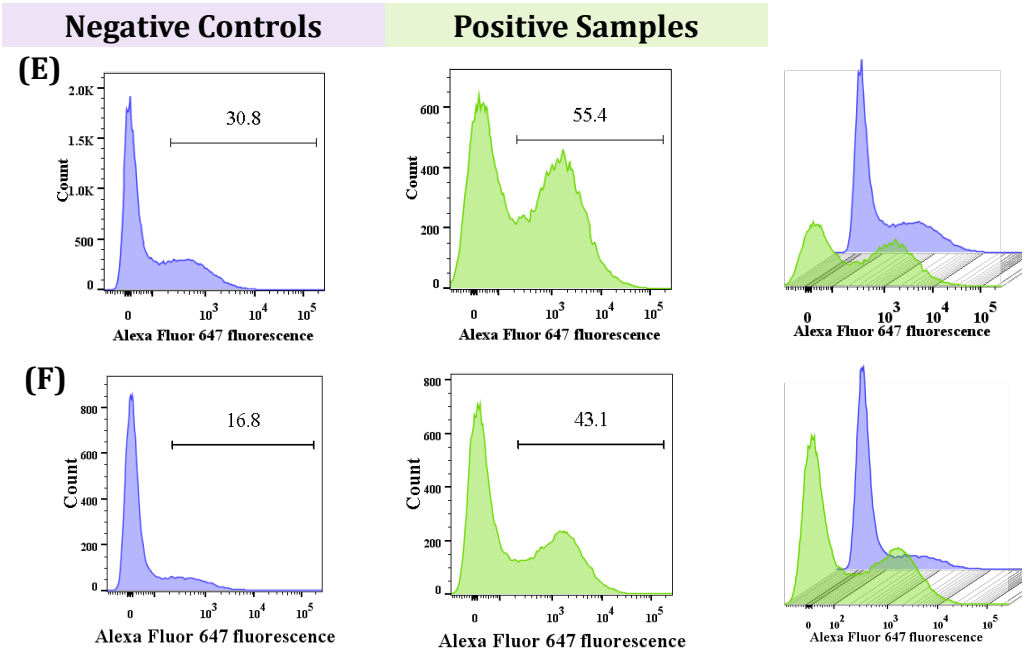


Fig. S1. Fluorescence intensity histograms of annamox sludge samples presenting effects of different chemicals and enzymes on the permeabilization of bacterial cell walls, especially for *Planctomycetes* cells, using the proposed in-solution Flow-CARD-FISH. (A) and (B), samples permeabilized using 0.2 and 0.5 mg·L^{-1} lysozyme, respectively. (C) and (D), samples permeabilized using 0.2 and 0.5 mg·L^{-1} proteinase K, respectively. (E) and (F), samples permeabilized using 10% and 20% SDS, respectively. Events inside the gates of histograms are detected as probe-labeled anammox bacteria. All samples presented were processed by the optimized TSA process of HRP labeling for 12 h at 20°C and fluorescent tyramide labeling for 30 min at 25°C using 5 μM oligonucleotide probe with a fluorescent substrate concentration of 1:50. The fluorescence intensity histograms of samples without permeabilization, which had extremely low fluorescent signal intensities close to the baseline noise, are not shown.

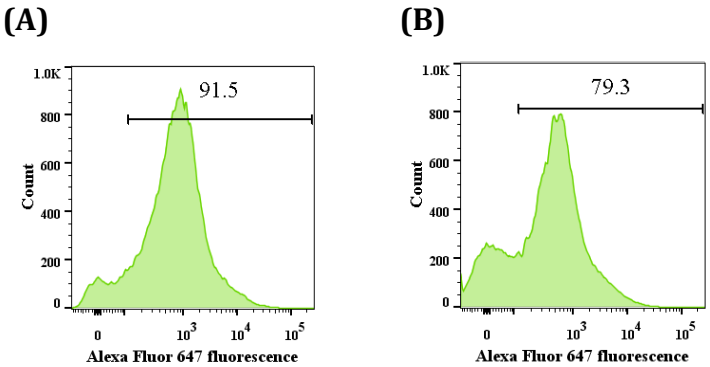


Fig. S2. Presence of false positives in negative controls caused by the endogenous peroxidase activity, which resulted in a severely overestimated anammox bacteria abundance by Flow-CARD-FISH. (A) and (B), fluorescence intensity histograms of negative controls incubated in peroxide solution for 30 and 60 min at 25°C, respectively.

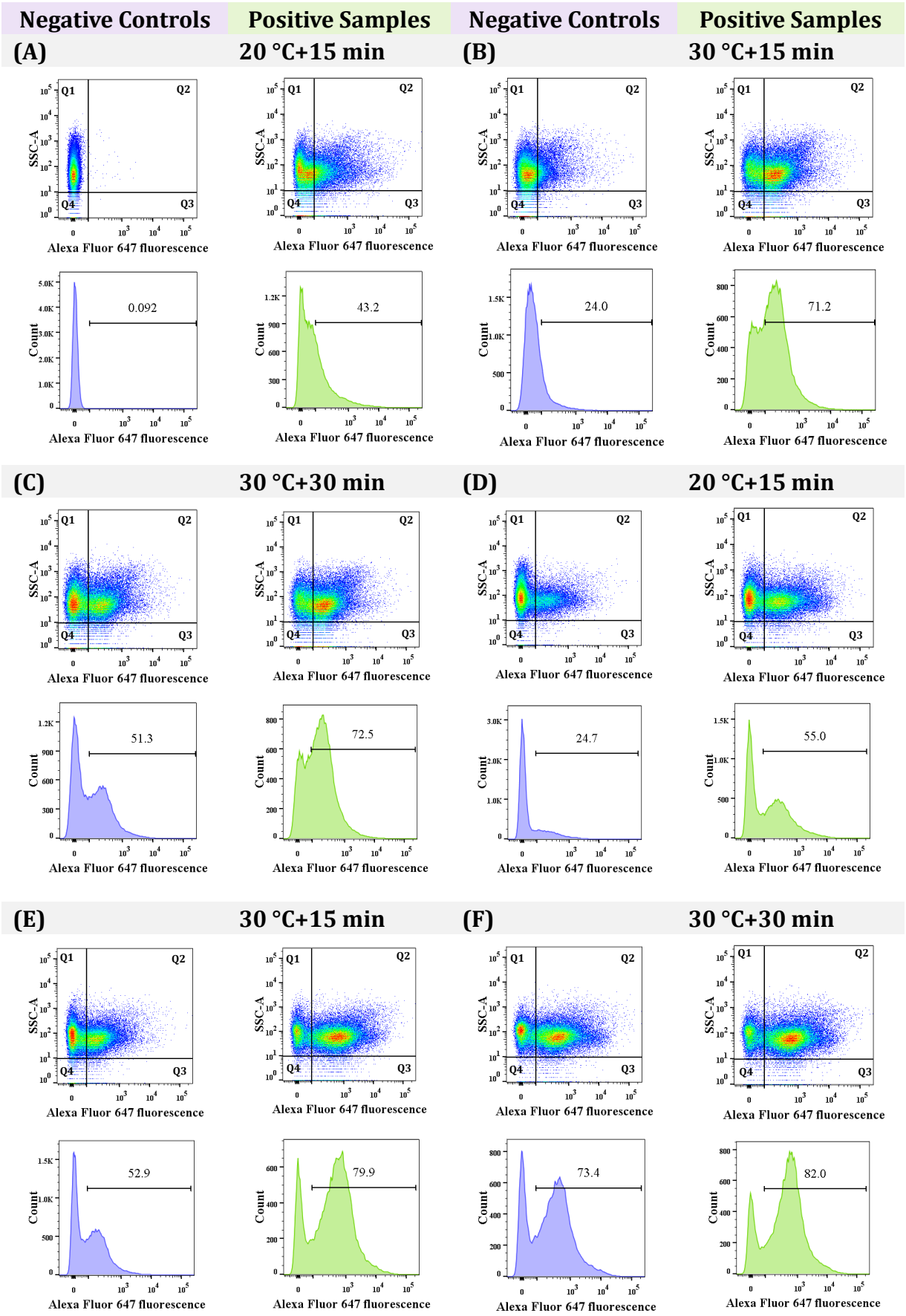


Fig. S3. Effects of HRP labeling temperature, fluorescent tyramide labeling duration and fluorescent substrate (Alexa Fluor 647 fluorophore) concentration on in-solution CARD-FISH. Events inside the gates of histograms were detected as probe-labeled anammox

bacteria (generally corresponding to gate Q2 in cytograms). (A)–(C), tyramide labeling with fluorescent substrate concentration at 1:100; (D)–(F), tyramide labeling with fluorescent substrate concentration of 1:50. (A) and (D): HRP labeling for 12 h at 20°C and fluorescent tyramide labeling for 15 min at 25°C; (B) and (E): HRP labeling for 12 h at 30°C and fluorescent tyramide labeling for 15 min at 25°C; (C) and (F): HRP labeling for 12 h at 30°C and fluorescent tyramide labeling for 30 min at 25°C. All samples presented were hybridized with oligonucleotide probes at concentrations of 5 µM. HRP labeling at 10°C for 12 h showed almost no signal enhancement in the positive samples (data not shown).

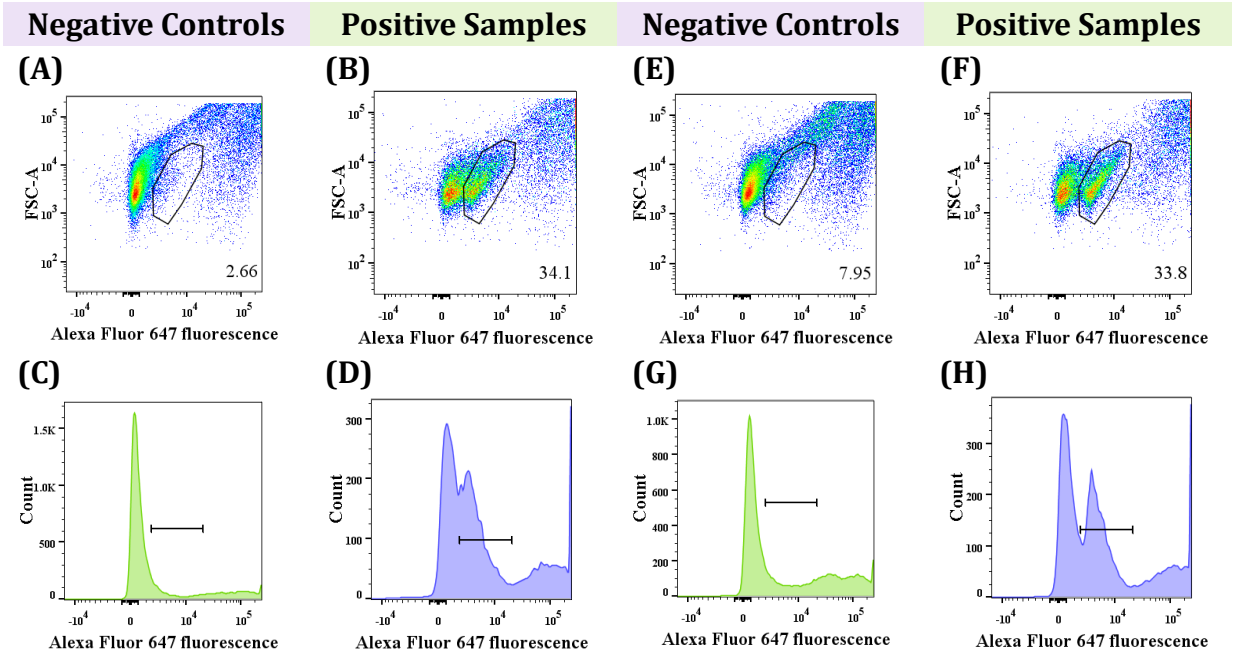


Fig. S4. Effects of fluorescent substrate (Alexa Fluor 647 fluorophore) concentration on CARD-FISH embedded in agarose. Cytograms of (A), (B), (E) and (F) and fluorescence intensity histograms of (C), (D), (G) and (H) analyzed by FCM. Events inside the polygon gate of cytograms are detected as probe-labeled anammox bacteria (generally corresponding to the gate in histograms). (A)–(D): fluorescence tyramide labeling with fluorescent substrate concentration of 1:50. (E)–(H): fluorescence tyramide labeling with fluorescent substrate concentration of 1:25. All samples presented were processed by the TSA process of HRP labeling for 12 h at 20°C and fluorescent tyramide labeling for 30 min at 25°C using 20 µM oligonucleotide probes.

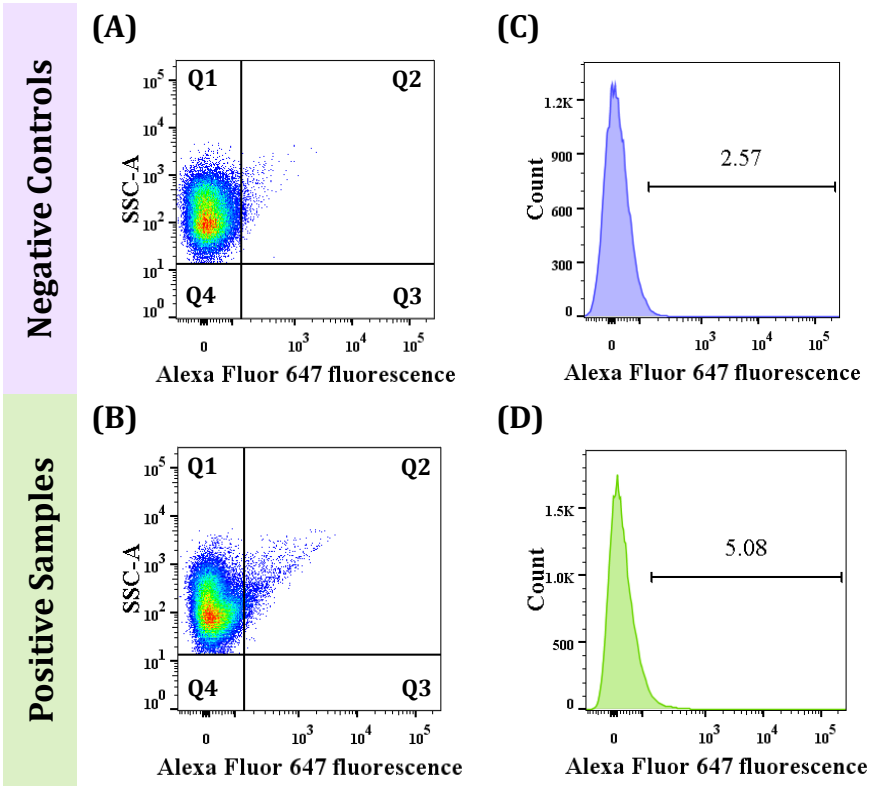


Fig. S5. Results of CARD-FISH embedded in agarose by adopting the optimized treatment used in the in-solution CARD-FISH. HRP labeling for 12 h at 20°C, fluorescent tyramide labeling for 30 min at 25°C; fluorescent substrate concentration of 1:50; 5 μM oligonucleotide probe. (A) and (B), cytograms; (C) and (D), fluorescent intensity histograms.

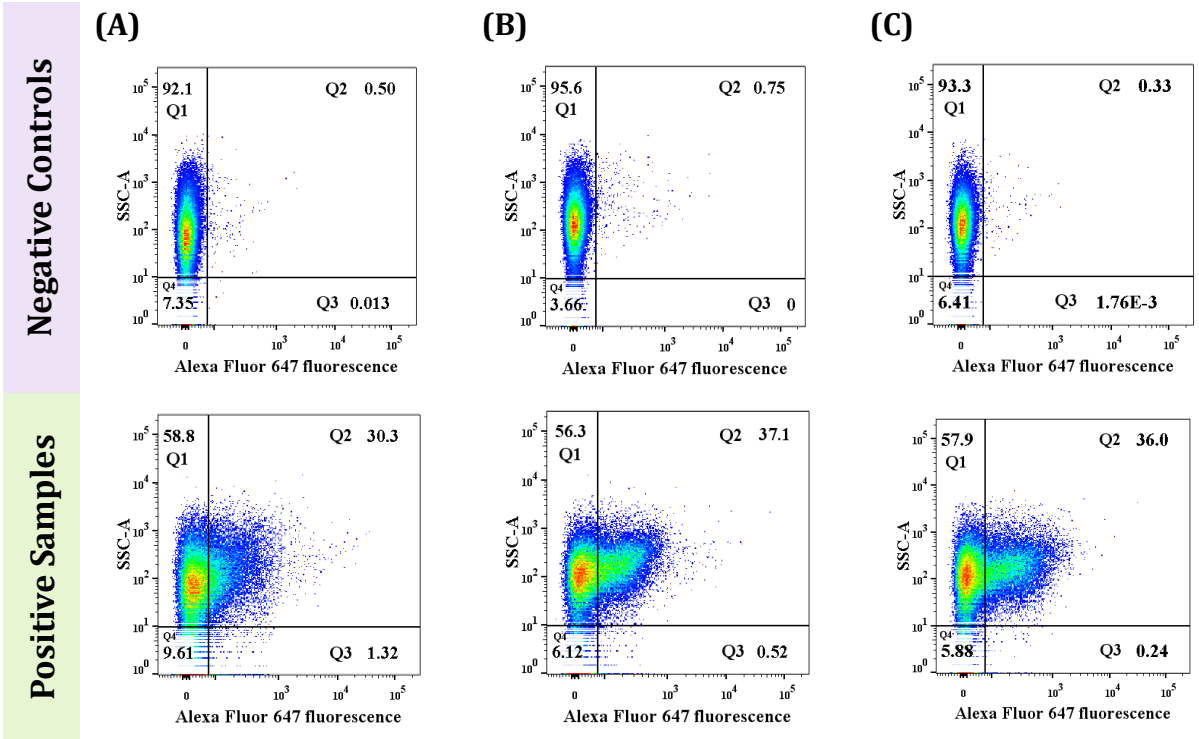


Fig. S6. Cytograms of samples hybridized by standard FISH with monolabeled probes (Flow-monolabeled-FISH). Events in the gate Q2 are targeted anammox bacteria. (A), Without permeabilization. (B) and (C), permeabilized with 0.2 and 0.5 mg·L^{-1} lysozyme, respectively.

Fig. S7. Optimized protocol and critical notes for anammox bacteria quantification by Flow-CARD-FISH.

Process and Methods	Notes
1. Fixation and homogenization	
1) Fix samples with paraformaldehyde (4%) at a ratio of 1:3 v/v at 4 °C for 12 h. 2) Resuspend in dispersant of Triton X-100 (5%) and sodium pyrophosphate (10 mM). 3) Sonicate with a sonication probe (20 kHz, 20 W) for 3 min.	a) Prevent prolonged fixation to avoid reduced bacteria counts. b) The optimal sonication transferred specific energy of 90 $kJ \cdot L^{-1}$ for anammox sludge with MLVSS of 3–5 $g \cdot L^{-1}$ is recommended.
2. Permeabilization and inactivation of peroxidases	
1) Incubate in lysozyme for permeabilization (≥12000 $U \cdot mL^{-1}$, 25°C, 60 min). 2) Incubate in H_2O_2 solution to inactivate endogenous peroxidase (final concentration of 3% in ultrapure water, 25°C, ≥ 90 min).	a) To process 50 µL sludge, 1 mL lysozyme/ H_2O_2 was employed. b) For completely inactivity the endogenous peroxidase, 3% H_2O_2 incubation for at least 90 min is indispensable.
3. Hybridization	
1) Resuspend in the mixture of 50 µL hybridization buffer and 20 µL probe stock solution (5 µM). Incubate at 46 °C for 2 h on a rocking plate. 2) Add 1 mL prewarmed (48 °C) washing buffer prior to centrifugation. 3) Incubate in 1 mL washing buffer at 48 °C for 15 min and rinse for three times with precooled (4 °C) ultrapure water to rinse excess probes.	a) The distilled water or ultrapure water in standard hybridization buffer must be replaced with blocking reagent (5% goat serum used in this study) to prevent non-specific false positive signals
4. Tyramide signal amplification	
1) Incubate in HRP conjugation streptavidin solution (1:50 in 1% blocking reagent) at 20°C for 12 h. 2) Wash for three times with PBSTx at 25 °C to rinse the excess HRP conjugation streptavidin. 3) Incubate in fluorescence substrate mix (1 part of fluorescence-labeled tyramide and 50 parts of amplification buffer) at 25 °C for 30 min. 4) Wash in the dark in sequence with PBSTx, ultrapure water, and 96% ethanol. Rinse with ultrapure water and 96% ethanol at 25 °C for 1 min to decrease background fluorescence.	a) To label 50 µL anammox sludge, 1 mL HRP conjugation streptavidin solution was employed. b) Adequately wash after HRP labeling by using 0.1% Triton X-100 amended PBS (PBSTx). c) 50 µL tyramide working solution was employed to label 50 µL sludge. d) Amplification buffer: 1×PBS, 0.0015% H_2O_2, 0.1% blocking reagent; or Tris-HCl buffer (pH = 7.4).
5. Flow cytometric analysis	
1) Disperse bacteria by sonication using an ultrasonic probe for 30 s (20 kHz, 20W) on ice and further diluted with filtered 1×PBS. 2) Counterstain with 20 µL DAPI (5 $\mu g \cdot mL^{-1}$) at 25 °C for 15 min in the dark. 3) Samples were submitted to FCM analysis within 2 h after resuspension and counterstaining. 4) Gate anammox bacteria populations according to the fluorescence signals, FSC and SSC.	a) The diluted bacteria suspensions containing about 10^{10} cells$\cdot L^{-1}$ is considered as optimal concentration for the flow cytometric analysis. b) The gate should be consistent.

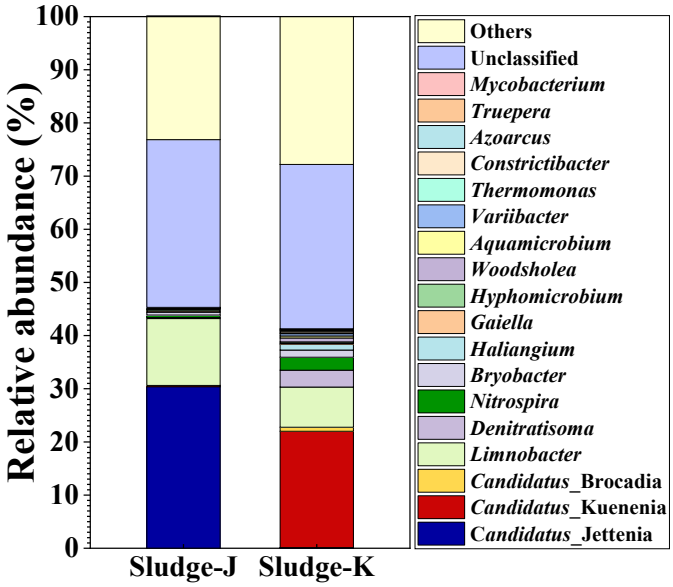


Fig. S8. Taxonomic distributions of the anammox sludge sample microbial communities at the genus level by 16S rRNA sequencing

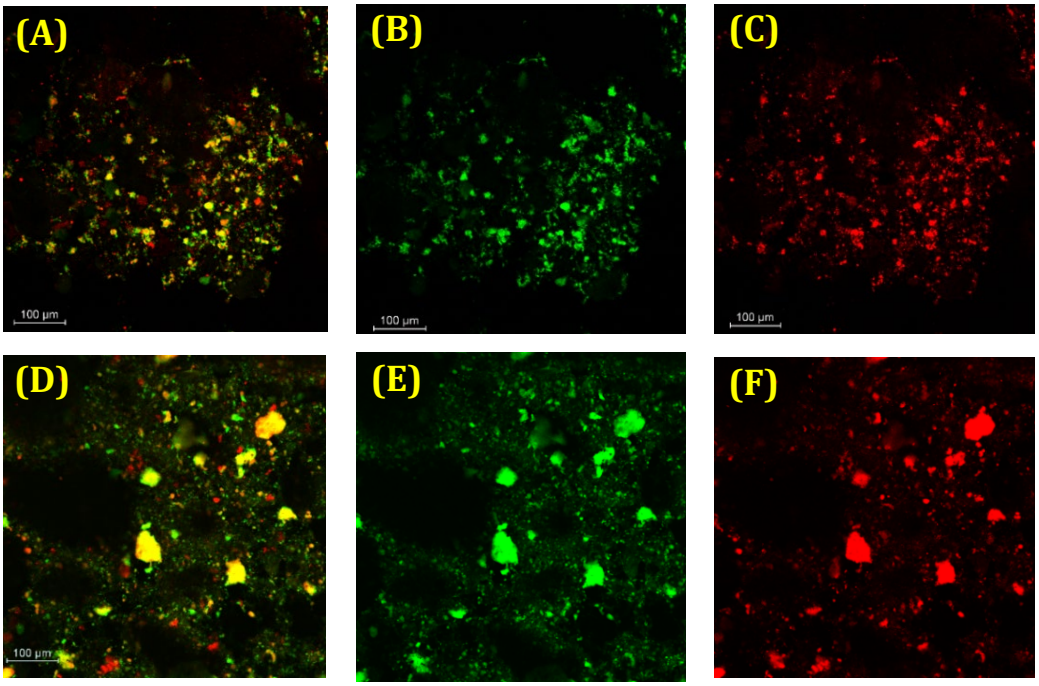


Fig. S9. Representative standard FISH-CLSM digital images illustrating the abundance of anammox bacteria in two anammox sludge samples. Sludge-J: (A)–(C); Sludge-K: (D)–(F). DAPI-stained bacteria (green) and Alexa Fluor 647-labeled AMX 368 probe (red, specific to anammox bacteria). Scale bars: 100 μm. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

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