

Supplementary Material

Specificity re-evaluation of oligonucleotidic probes for the detection of marine picoplankton by Tyramide Signal Amplification-Fluorescent *in situ* Hybridization

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Supporting data: *Symbiomonas scintillans* RCC257 18S ribosomal RNA gene, source = RCC. Highlighted zones indicate the binding sites for Pela01 (dark blue), Chlo02 (light blue) and NChlo01 (grey), with mismatches or deletions highlighted in red.

5'CTCGTAGTTGAACTTCTGAGCCACGTGCATGGGCGCCACACATGTGGCCCCTCA
CTGTGGCTCATCTTCGTTGTTGAACCTTGGCGGGCGCAAGTCACCTTGGGGATCGG
CGTCATTTACTGTGAAAAAATTAGAGTGTTC AAGGCAGGCGTTAGCCCGTATCCA
CTAGCATGGAATAATAAGATAGGACCTCGTGCTATTTTGTGGTTGCGGTTTCGAG
GTAATGATTAATAGGGACAGTTGGGGGTATTCGTATTTAGCTGTCAGAGGTGAAA
TTCTTAGATTTGTTAAAGACGAACGACTGCGAA **AGCATCTACCAAGGATGT**TTTC
ATTAATCAAGAACG **AAAGT** **CAGGGG** **ATCGAAG**AGGATTAGATACCCTCGTAGTC
CTGTACCATAAACGATACCGACCCGCGATTGTCAGGTGTATTCAAGACCCTGGCA
GCAGCGAAGAAGAAATTCCGAGTCTTTGGGTTCCGGGGGGAGTATGGTTGCAA
GCTGAAACTTAAAGGAATTGACGGAAGGG **CACC** ***CAAG** **AAGTGGAGC**CTGCGGC
TTAATTCGACTCAACACGAGAAACTTACCAGGTCCGGACAAAACAAGGATTGA
CAGTTTGAGAGTACTTTCTTGATTTTTTGGTTGGTGGTGCATGGCCGTTCTTAGTT
GGTGGAGTGATTTGTCTGGTTGATTCCGATAACGAACGAGACCTTCGCCTGCTAA
TTAGTTCTCTCCCCTACCGGGGTATTGAGGACTTCTTAGAGGGACTCGTT-3'

Cell cultures

Haloarcheon msnc14(3) was grown on the medium defined by Tapilatu *et al.* (2010), in which the agar was omitted. All bacterial media were supplemented with 15 g L⁻¹ agar and prepared in distilled water according to the DMSZ instructions: medium 545 was prepared from commercial Tryptic Soy Broth (Sigma #22092), medium 514 from bacto marine broth (DIFCO 2216), while media 621 (PYGV AGAR), 607 (M13 *Verrucomicrobium* Medium), 762 (*Halomonas desiderata* Medium: Glucose 5.0 g L⁻¹ MgCl₂ x 6 H₂O 0.2 g L⁻¹, KH₂PO₄ 1.0 g L⁻¹, KNO₃ 2.0 g L⁻¹, Na₂CO₃ 5.4 g L⁻¹, NaHCO₃ 4.2 g L⁻¹, final pH 9.5-10.0), were prepared by assembling the different chemicals. The eukaryotic L1-Si and K media were prepared from commercial kits, and the « Riz » (Rice) medium was provided by the RCC.

Sample preparation

The 10% buffered paraformaldehyde (PFA, w:vol) stock solution was prepared by adding 10 g of PFA to 65 mL warm (56°C) sterile MilliQ water under stirring. Dissolution was obtained by warming and adding few drops of concentrated NaOH (10N) to obtain a translucent solution. The solution was then removed from the heating plate, 10 mL of 10% Phosphate Buffered Saline pH 7.4 was added, and pH was adjusted to 7.2, before adjusting the volume to 100 mL with sterile MilliQ water and filtering the preparation on chemistry paper filter. Aliquots of 10 mL stock 10% PFA were stored at -20°C up to 1 year or at 4°C for up to 3 weeks to avoid formaldehyde dismutation.

Strain RCC375 and all bacterial strains were vortexed after fixation to disaggregate clumps of cells. After this step, the fixed cells may be quickly frozen at -80°C until further processing. Freshly fixed cells or frozen aliquots (limited to one freeze-thaw cycle) were then collected onto 0.2 µm-pores 47 mm PCTE filters at a concentration allowing the detection of 10-50 cells per camera field (depending on the size of the cells). The cells were further embedded on the filter in low-gelling point 0.4% agarose ([wt/vol] in Milli-Q water), dried at 37°C for 15 min, dehydrated for 10 min in 100% ethanol and quickly frozen at -80°C until further analysis.

Cell perforation and hybridization by TSA-FISH

Picoeukaryotes did not need any perforation treatment for the penetration of the HRP. Bacterial cell walls were perforated as in Biegala & Raimbault (2008), with slight modifications, using a 60 min treatment at 37°C with $\approx 500,000$ U mL⁻¹ lysozyme (10 mg mL⁻¹, ref. L6876 $\approx 40,000$ U mg⁻¹) in 0.05 M Na₂EDTA [pH 8.0], 0.1 M Tris-HCl [pH 7.5]. It was followed by three rinsing steps of 5 min in 5 mL Milli-Q sterile water. Then, a 30 min incubation at 35°C with 60 U mL⁻¹ achromopeptidase (ref. A3547 $\approx 4,500$ U mg⁻¹, Sekar *et al.*, 2003) in 0.01 M NaCl, 0.1 M Tris-HCl [pH 7.5], was followed by three rinsing steps of 5 min in 5 mL Milli-Q sterile water.

The perforation of archaeal cell walls was done as in Teira *et al.* (2004) for 60 min at 37°C with 4 U mL⁻¹ proteinase K (6.7 µL mL⁻¹ of a 600 U mL⁻¹ solution, ref. P4850) in 0.05 M Na₂EDTA, 0.1 M Tris-HCl [pH 8.0] (Teira *et al.*, 2004). After this treatment, filters were rinsed three times in 5 mL sterile Milli-Q water, incubated for 20 min at room temperature in 5 mL 0.01 M HCl and washed twice with 5 mL Milli-Q water.

Before the hybridization step, 1:16 filter portions were soaked for 10 min in 100% ethanol and dried 15 min at 37°C. The hybridizations with 5'-horseradish peroxidase (HRP)-labeled oligonucleotide probes (5 ng DNA µL⁻¹, final oligonucleotide concentration) using the formamide concentrations detailed in Table 2, were performed for 2h at 35°C as in Biegala *et al.* (2002), except for *Haloarcheon* msnc14(3), which was incubated overnight with Arch915 (Stahl and Amman, 1991, 5% formamide, Ushio *et al.*, 2013), with Euk1209 or without probe as a "no probe control". After the hybridization, filters were washed twice for 30 min at 37°C in 5 mL of prewarmed washing buffer consisting of 0.02 M Tris-HCl [pH 7.5], 0.01% Sodium Dodecyl Sulfate, 5 mM Na₂EDTA [pH 8] and NaCl concentrations listed in Table S2 (according to Amann and Schleifer, 2005). Equilibration in Tween-NaCl-Tris buffer (TNT), TSA reaction and TNT rinsing steps were done as in Biegala *et al.* (2002), using the TSA Plus Fluorescein Evaluation Kit (green fluorescent emission). Filters were then allowed to

equilibrate for 10 min at room temperature in sterile 1X PBS [pH 7.4], their down-side was wiped dry before DNA staining as in Biegala *et al.* (2002) on eubacteria and eukaryotes, for 15 min in the dark by 10 μL of a 5 $\mu\text{g mL}^{-1}$ DAPI (4',6-diamidino-2-phenylindole dihydrochloride, blue fluorescent emission) mixture consisting in two volumes of 10 $\mu\text{g mL}^{-1}$ aqueous DAPI solution, one volume of 1X PBS and one volume of AF1 (Citifluor Ltd.). After a 10 min washing step in sterile 1X PBS [pH 7.4], filters down-side were wiped dry, and they were mounted in AF1 anti-fading reagent (Citifluor Ltd.), and stored at 4°C until further microscopic analysis.

Microscopy

Images were acquired with an epifluorescence *ECLIPSE 50i* microscope (Nikon) equipped with an Halogen lamp (6V 30W, H65761, Orbitec), two objectives (40X: NA 0.75N Plan Fluor WD 0.72mm, Nikon; and 100X: NA 1.30N Plan Fluor WD 0.16mm, Nikon), excitation (ex.) and emission (em.) dichroic filters (365 \pm 10 nm ex., 400 nm em. for DAPI detection; 480 \pm 40 nm ex., 510 nm long pass em. for the Fluorescein IsoThioCyanate FITC detection) and a digital camera (QICAM 12-bit color cooled, QImaging). All the pictures were taken at 100X magnification, except for *L. reticulosa* (40X).

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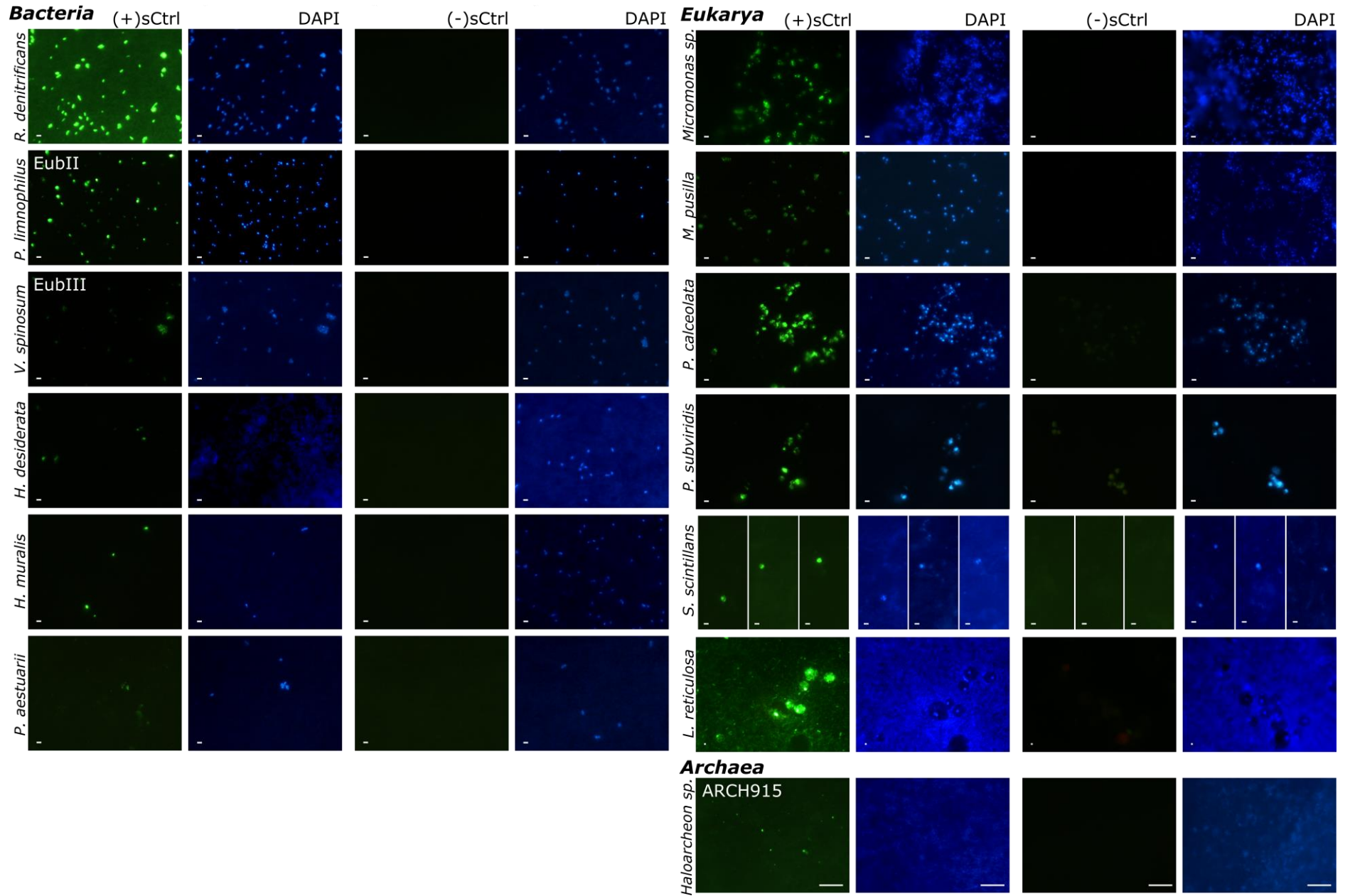
Table S1: *In silico* analysis of the probes. The number of sequences presenting 0-3 mismatches are displayed, with the mismatch location (c = central, t = terminal, (-) = not analysed). For probe mixes, sequences with 1 mismatch were only counted if they had > 0 mismatch with all the probes. Dark background indicates the limit of the probe specificity. (*Italics*) = *unidentified organisms*, ^A Eilers *et al.*, 2001, ^B Not *et al.*, 2002, ^C Not *et al.*, 2004, ^D *This study*.

Probe Name	Target	Database	0 mismatch		1 mismatch		2 mismatches		3 mismatches	
			Target	Out group	Target	Outgroup	Target	Outgroup	Target	Outgroup
Eub338	16S rRNA, <i>Bacteria</i>	SSU Ref 114	572724	2	1227 _t 22352 _c	30 _c	781 _{tc/tt} 13979 _{cc}	28 _{tc} 745 _{cc}	-	-
EubII	16S rRNA, <i>Bacteria</i>	SSU Ref 114	3699	0	13 _t 10011 _c	0	7082	22 _c	577259	118
EubIII	16S rRNA, <i>Bacteria</i>	SSU Ref 114	9915	0	41 _t 4287 _c	22 _c	576984	118	-	-
Eub338+EubII+EubII I	16S rRNA, <i>Bacteria</i>	SSU Ref 114	586338	2	1281 _t 22811 _c	30 _c	-	28 _{tc} 745 _{cc}	-	211 _{ttc}
Gam42a	23S rRNA, <i>Gammaproteobacteria</i>	LSU Parc 114	6639	43 (83)	31 _t 939 _c	1129 _c (4 _t 334 _c)	3 _{tt} 21 _{tc} 72 _{cc}	44 _{tc} 143 _{cc} (6 _{tc} 51 _{cc})	16 _{ttc} 3 _{ccc}	2 _{ttc} 164 _{ttc} 480 _{ccc} (26 _{ttc} 128 _{ccc})
Gam42a + comp Bet42a	23S rRNA, <i>Gammaproteobacteria</i>	LSU Parc 114	6639	43 (83)	31 _t 353 _c	102 _c (4 _t 106 _c)	-	-	-	-
Ros537 ^A	16S rRNA, <i>Roseobacter</i> clade	SSU Ref 114	4935 (1443)	51 (141)	29 _t 296 _c (540 _c)	5 _t 5145 _c	1 _{tt} 91 _{tc} 88 _{cc} (76 _{tc} 221 _{cc})	23733 _{tc} 7142 _{cc}	-	-
Roseo536R	16S rRNA, <i>Roseobacter</i> clade	SSU Ref 114	4935 (1438)	51 (141)	29 _t 296 _c (535 _c)	2 _t 5145 _c	1 _{tt} 7 _{tc} 172 _{cc} (6 _{tc} 291 _{cc})	572 _{tc} 30482 _{cc}	-	-
+ comp	16S rRNA, <i>Roseobacter</i> clade	SSU Ref 114	4935 (1438)	51 (141)	29 _t 45 _c	2 _t 15 _c	-	-	-	-
Euk1209	18S rRNA, Eukaryotes	SSU Ref 113	61525	0	1272 _t 4223 _c	0	794	2	200	74
Chlo01	18S rRNA, green lineage	SSU Ref 114	3918	5396	4692	46939	95	7827	-	-
Chlo02	18S rRNA, green lineage	SSU Ref 113	8099	7	39 _t 337 _c	3544 _c	2 _{tt} 5 _{tc} 423 _{cc}	88 _{tc} 21227 _{cc}	-	-
NChlo01	18S rRNA, non- Chlorophyta	SSU Ref 113	44968	111	1182 _t 11169 _c	3190 _c	159 _{tt} 843 _{tc} 2317 _{cc}	1 _{tt} 55 _{tc} 1388 _{cc}	-	-
Chlo01+NChlo01 ^B	18S rRNA, Euk.	SSU Ref	54097	3	1416 _t	272 _c	-	-	-	-

	phytoplankton	113			7117 _c						
Chlo02+NChlo01	18S rRNA, Euk. phytoplankton	SSU Ref 113	49132	0	288 _t 9805 _c	45 _c	-	-	-	-	-
Euk1209+Chlo02	18S rRNA, Euk. phytoplankton	SSU Ref 113	61747	0	1256 _t 4380 _c	0	-	-	-	-	-
Euk1209+NChlo01	18S rRNA, Euk. phytoplankton	SSU Ref 113	64956	0	1181 _t 3016 _c	45 _c	-	-	-	-	-
Euk1209+NChlo01+ Chlo01 ^C	18S rRNA, Euk. phytoplankton	SSU Ref 113	65588	3	1198 _t 2481 _c	272 _c	-	-	-	-	-
Euk1209+NChlo01+ Chlo02 ^D	18S rRNA, Euk. phytoplankton	SSU Ref 113	65065	0	1273 _t 3239 _c	45 _c	-	-	-	-	-
Pela01	18S rRNA, Pelagophytes	SSU Ref 114	42	0	1 _c	0	1 _{cc}	7 _{ct}	0	2 _{tcc}	1006 _{ccc}
Pras04	18S rRNA, <i>Mamiellophyceae</i>	SSU Ref 114	390	3	1 _t 17 _c	8 _t 860 _c	0	2777 _{tc} 2322 _{cc}	-	-	-

Table S2: Strains used in this study, with the corresponding size and culture conditions (n.r. = not required). ^a RCC: Roscoff Culture Collection, France; CCMP: Provasoli-Guillard National Center for Culture of Marine Phytoplankton, USA; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany. ^b 12h:12h cycle.

Domain	Class	Strain ^a	Cell size (µm)	Culture Medium	Light intensity ^b (µE m ⁻² s ⁻¹)	Temperature (°C)
Eukarya	Prasinophyceae	<i>Micromonas sp.</i> CCMP2099	1-3	L1-Si	40	4
		<i>Micromonas pusilla</i> RCC299	2-3	K	145	20
	Pelagophyceae	<i>Pelagomonas calceolata</i> CCMP1214, RCC100	1-4	K	145	20
		<i>Pelagococcus subviridis</i> CCMP1429	2-4	L1-Si	350	15
		<i>Bicosoecid</i> <i>Symbiomonas scintillans</i> RCC257	2-3	Riz	4	20
Bacteria	Chlorarachniophyceae	<i>Lotharella reticulosa</i> RCC375	9-16	K	100	20
	Planctomycetacia	<i>Planctomyces limnophilus</i> DSM3776	1.1-1.5	621 agar	n.r., 500	28
		Verrucomicrobia	<i>Verrucomicrobium spinosum</i> DSM4136	0.8-3.8	607 agar	n.r., 500
	Gammaproteobacteria	<i>Halomonas desiderata</i> DSM9502	0.4-2.6	762 agar	n.r., 500	28
		<i>Halomonas muralis</i> DSM14789	0.4-3.0	514 agar	n.r., 500	28
	Alphaproteobacteria	<i>Roseobacter denitrificans</i> OCh114, DSM7001	0.6-2	514 agar	n.r., 500	20
		<i>Paracoccus aestuarii</i> B7, DSM19484	0.8-2.0	545 agar	n.r., 500	28
		Archaea	<i>Haloarcheon</i> <i>Haloarcheon</i> msnc14(3)	Pleomorphic 1-3	SCM liquid	n.r.



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Figure S1: Control hybridizations of the strains. (+)sCtrl : Showing that all strain studied had a potential of positive hybridization with a domain probes from Eubacteria, Eukaryote and Archea, using 50%, 40% and 5% formamide concentration respectively. (-)sCtrl: showing the potential of strains autofluorescence when omitting the probe in hybridization procedure (no probe control).. Scale bars indicate 2 μ m.

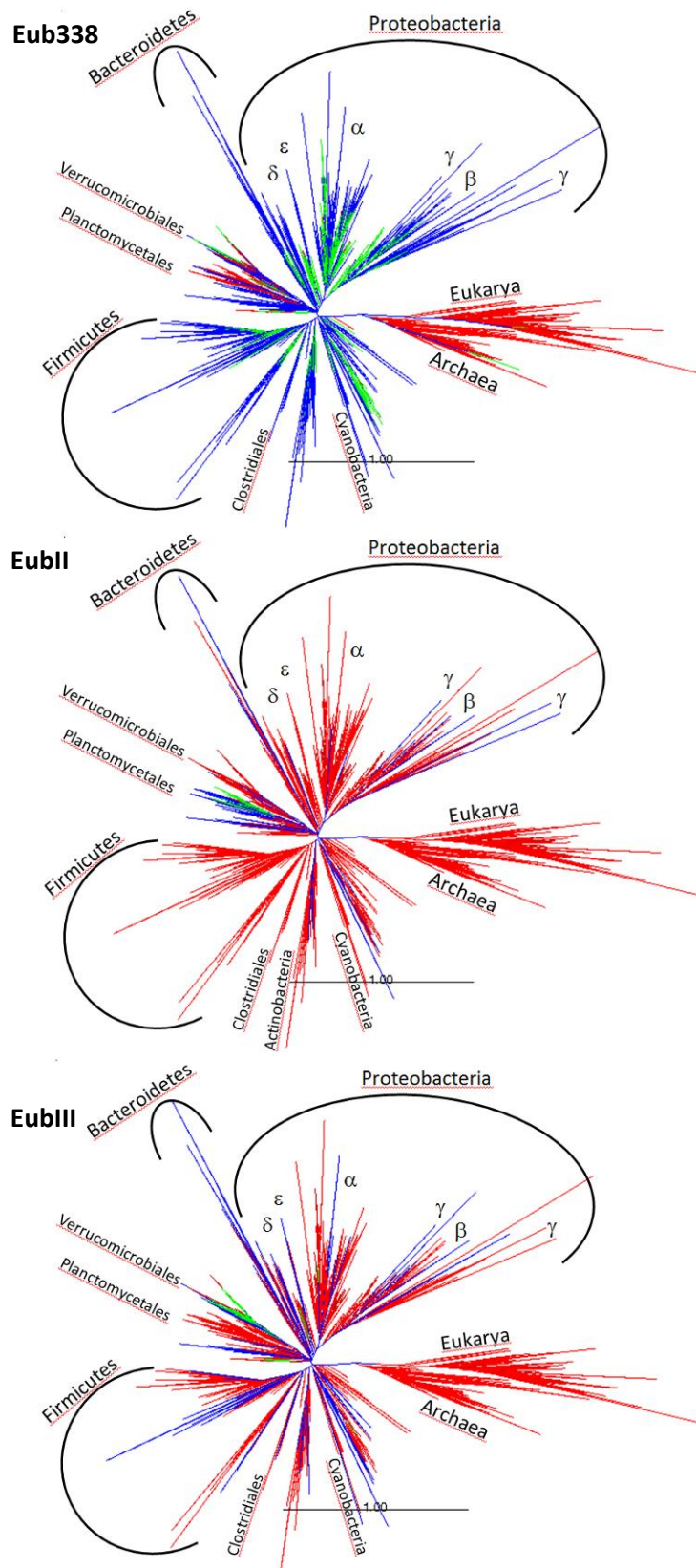


Figure S2: Representation of the current Eub338, EubII and EubIII probes specificity onto a phylogenetic tree calculated from the 739,633 DNA sequences encoding the small subunit ribosomal RNA available in the curated Silva SSU ref #111 database. Blue and Green, targets with 0 mismatch. Red, not targeted.

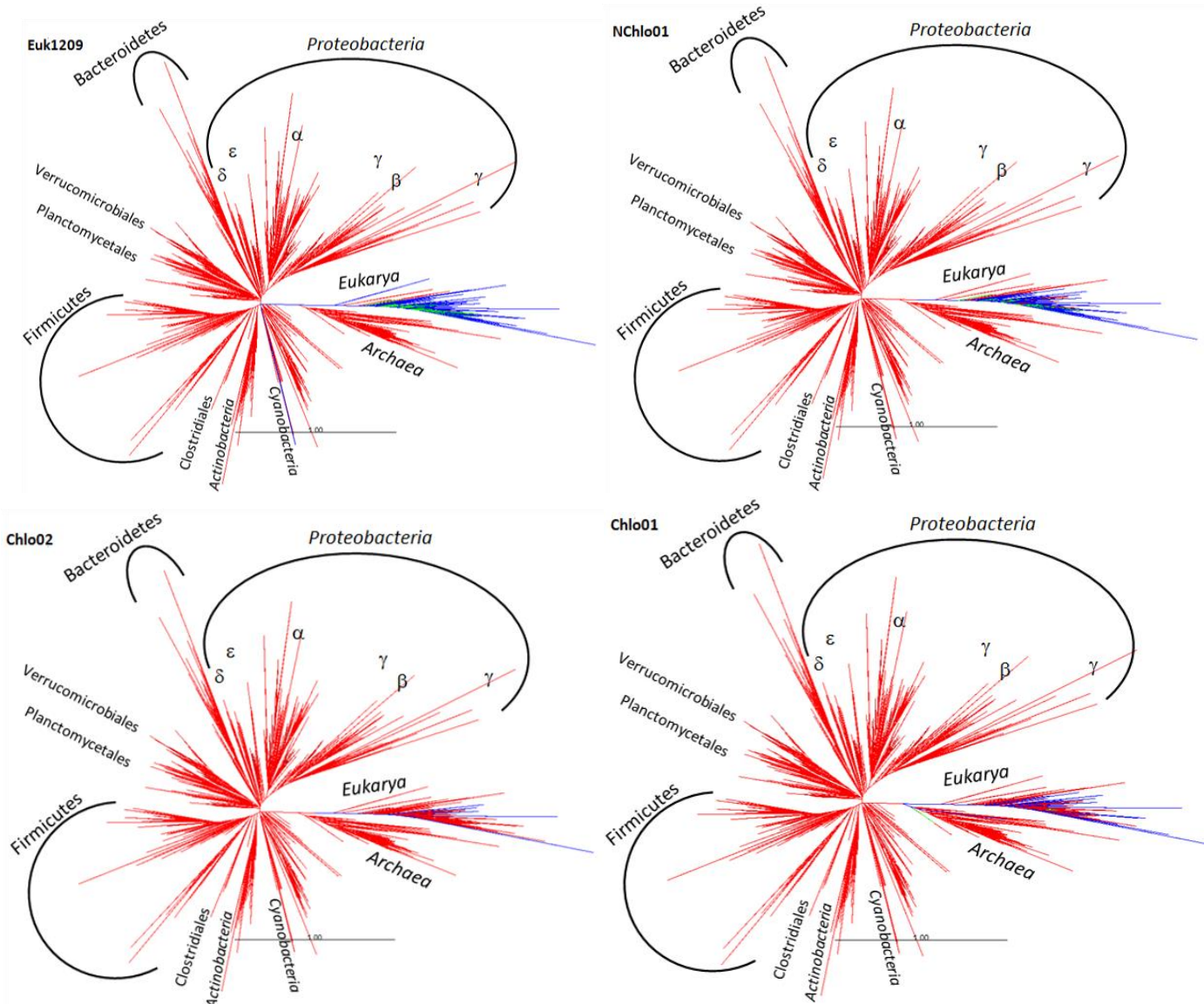


Figure S3: Current Euk1209, NChlo01, Chlo02 and Chlo01 probes specificity onto a phylogenetic tree calculated from the 739,633 DNA sequences available in the curated Silva SSU ref #111 database. Blue and Green, targets with 0 mismatch. Red, not targeted.