**S1 file: Dechloromonas probe analysis**

**Probe design and validation**

Probes were designed and evaluated with the ARB software package. The mathFISH software was used to calculate hybridization efficiency predictions for perfect and mismatched probe binding. The potential for non-target sequences with indels was also assessed. Competitor probes were designed for single base mismatched non-target sequences. Probe validation and optimization were based on empirically determined formamide dissociation curves where average fluorescent intensities of at least 50 cells calculated with ImageJ software were measured for varied hybridization buffer formamide concentration [FA] from 0-65% (v/v) (Fig. S1). Target and non-target axenic cultures were used for probe optimization where available, otherwise activated sludge was used (see Tab. S1).

**Results**

As the Dechlo2 probe was originally designed for use in microarrays, it was optimized for FISH and found to have an optimal [FA] of 40% [v/v] (see Fig. S1a). An additional probe, Dech219, was designed by shifting the target site 8 nucleotides and optimized to target the Dechloromonas group (Tab. S1 & Fig. S1b). Both the Dechlo2 and Dech219 target a broader phylogenetic cluster that includes the genus Ferribacterium, as well as some related unclassified clusters (see Tab. S2 and Fig. S2), with good coverage (Tab. S3). Application of these probes to activated sludge gave fluorescence for rods and cocobacilli, typically present in loose clusters within the flocs. Given the overlap of their target site, the Dechlo2 and Dech219 probe overlap in situ could not be directly assessed, but differences were not apparent when visually assessed in several full-scale sludges (data not shown).

Although slightly higher coverage is indicated for the Dech219 probe, the Dechlo2 probe has a number of target group weakly mismatched sequences that still give a positive signal, as demonstrated for Dechloromonas sp. R-28400 and Dechloromonas denitrificans (see Fig. S1a and Tab. S4). When considering calculated weak terminal mismatched sequences, the Dechlo2 probe gives slightly higher predicted coverage and specificity (data not shown) and is thus recommended for use.

Interestingly, all cells hybridizing the Dechlo2 and Dech219 probes also hybridized with the Nso190 probe, targeting Nitrosomonas-Nitrospira-related ammonia-oxidizing organisms, despite no evident in silico overlap between the two. Investigations were carried out to determine which of the probe sets was mis-hybridizing to non-target organisms. Single base mismatched Nitrospira-related sequences were detected with ‘Probe Match’ (RDP II) (Tab. S4). However, these sequences were all partial (<1200 bp), from soil, and almost entirely generated by a single study. The single base mismatch is the same for both Dechlo2 and Dech219 probes (E. coli position 223). In addition, application of unlabelled competitor probes specific to this mismatch made no evident difference to binding of the Dechloromonas probes in the activated sludge samples tested. Few Dechloromonas-related sequences have a single base mismatch with the Nso190 probe, although almost all have 2 terminal mismatches (Tab. S4). Application of an unlabelled competitor probe specific for these mismatches always eliminated in situ crossover between the Nso190 and Dechloromonas probes. This was confirmed by applying the probe to D. agitata, which gave bright positive fluorescence at 40% [FA] and slightly higher than background levels at 55%. These analyses indicate that it is the Nso190 probe that is mis-hybridizing with Dechloromonas-related organisms giving the overlap in in situ coverage. Since the initial description of the Nso190 probe suggested its application at 55% [FA], several studies have suggested lower concentrations, ranging from 20-50% [FA], be used due to low and inconsistent
Since signal was detected when the Nso190 probe was applied to D. agitata all the way up to 55% [FA], it is recommended that the competitor probe designed here be used, regardless of the [FA] selected, to prevent the probe from mishybridizing to Dechloromonas-related organisms.

References:


3. McIlroy S.J., Tillett D., Petrovski S., Seviour R.J. Non-target sites with single nucleotide insertions or deletions are frequently found in 16S rRNA sequences and can lead to false positives in fluorescence in situ hybridization (FISH). Environ Microbiol 2011; 13; 38-47.


